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(57) Abstract

The present invention describes the design, synthesis and therapeutic use of a variety of novel inhibitors of polyamine transport. The main feature of this class of transport inhibitors is to incorporate a linker or side chain that prevents the uptake of polyamines and helps to conjugate polyamine analogs to form dimers with high inhibitory potency against polyamine uptake. These new compounds incorporate features that are designed to maximize their chemical and metabolic stability and their ability to bind to the polyamine transporter, and to minimize their toxicity by preventing their absorption by the cells. The purpose of such inhibitors is to prevent the uptake or salvaging of circulating polyamines by rapidly proliferating cells such as tumor cells, in order to potentiate the effect of therapeutic inhibitors of polyamine biosynthesis such as Eflornithine.

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INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 456 908 A (AZIZ SHEWAN M ET AL) 10 October 1995 cited in the application see the whole document ---	1
P,X	M. HUBER ET AL.: "2,2'-Dithiobis(N-ethyl-spermine-5-carboxamide) is a ..." J. AMER. SOC. BIOCHEM. MOL. BIOL., vol. 271, no. 44, 1 November 1996, pages 27556-27563, XP002060777 see the whole document --- -/--	13-15

[X] Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	A. ASK ET AL.: "Antileukemic effect of..." CANCER LETTERS, vol. 69, 1993, pages 33-38, XP002060778 see the whole document ---	1
A	S. AZIZ ET AL.: "A novel polymeric spermine conjugate..." J. PHARMACOL. EXP. THER., vol. 274, no. 1, 1995, pages 181-186, XP002060779 see the whole document -----	1

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POLYAMINE TRANSPORT INHIBITORS

The present application claims priority to U.S. Serial No: 08/735,130 filed 22 October, 1996.

FIELD OF THE INVENTION

The present invention relates to a novel class of competitive inhibitors of natural polyamine transport in mammalian cells. The present invention is more particularly directed to low molecular weight, high-affinity, specific, impermeant, pure antagonists of polyamine transport of a structure different to that of endogenous polyamines. The novel inhibitors of the present invention exhibit an effect on cultured tumor cells essentially cytostatic, with minor non-specific effects. The present invention is also directed to the use of such novel inhibitors of polyamine transport to evaluate the antitumor efficacy of polyamine depletion strategies with minimal systemic cytotoxic effects or to control and treat disorders involving unrestrained cell proliferation and/or cell differentiation wherein polyamine transport is required.

BACKGROUND OF THE INVENTION

Natural polyamines such as putrescine (1,4-butane-diamine), spermidine (*N*-3[-aminopropyl]-1,4-diaminobutane) and spermine (*N,N*[1]-bis-[3-aminopropyl]-1,4-butane-diamine) play essential roles in the control of macromolecular synthesis and growth processes in eukaryotic cells. Cells maintain appropriate polyamine concentrations principally by *de novo* synthesis from amino acids wherein ornithine decarboxylase catalyzes conversion of ornithine to putrescine, which is then converted to spermidine and spermine. Most tissues also possess a specific plasma membrane transport system allowing for utilization of plasma sources of polyamines.

Inhibitors of polyamine biosynthesis such as a-difluoromethylornithine (DFMO), which inhibits ornithine decarboxylase, cause an extensive depletion of polyamines followed by growth arrest in virtually all known mammalian cell types *in vitro*. Since tissues such as tumor cells and other transformed or rapidly proliferating cells exhibit a high demand for polyamines, these properties have encouraged an extensive assessment of

such compounds for the treatment of proliferative diseases, including several types of tumors, in experimental models and in clinical trials. Unfortunately, the antitumor efficacy of such inhibitors *in vivo* has been disappointing. The failure of DFMO to halt tumor growth in animal models has been clearly correlated with the elevated polyamine transport activity found in transformed cells. Indeed, decontamination of the gastrointestinal tract, which is the main vector of circulating polyamines through bacterial microflora activity, along with a polyamine-free diet, markedly potentiate the *in vivo* efficacy of DFMO against tumor progression. Moreover, mutant mouse leukemia cells deficient in polyamine transport are much more susceptible than the parental strain to growth inhibition by DFMO treatment in host animals. Besides, growth inhibition associated with DFMO-induced polyamine depletion in ZR-75-1 human breast cancer cells can be completely reversed by concentrations of spermidine as low as 300 nM, i.e., such as those found in human plasma (Moulinoux, J.-P., Quemener, V., and Khan, N.A. 1991. *Cell. Mol. Biol.* **37**: 773-783; Scalabrino, G. and Ferioli, M.E. 1981. *Adv. Cancer Res.* **36**: 1-102; Bachrach, U., 1989. in *The Physiology of Polyamines* (Bachrach, U. and Heimer, Y.M., eds.) Vol. II, pp. 235-249, 2 vols, CRC Press, Boca Raton, FL). The striking efficiency of the transport system to salvage exogenous polyamines in DFMO-treated cells owes to its upregulation consecutive to polyamine depletion (Seiler, N. and Dezeure, F. 1980. *Int. J. Biochem.* **22**: 211-218; Byers, T.L. and Pegg, A.E. 1990. *J. Cell Physiol.* **143**: 460-467; Lessard, M., Zhao, C., Singh, S.M. and Poulin, R. 1995. *J. Biol. Chem.* **270**: 1685-1694; Kakinuma, Y., Hoshino, K., and Igarashi, K. 1988. *Eur. J. Biochem.* **176**: 409-414). These data reinforce the view that cellular import of exogenous polyamines is the main factor limiting the efficacy of DFMO and other polyamine biosynthesis inhibitors as antitumor agents *in vivo* (Sarhan, S. Knödgen, B., and Seiler, N. 1989. *Anticancer Res.* **9**: 215-224; Hessels, J., Kingma, A.W., Ferwerda, H., Keij, J., Van der Berg, G.A., and Muskiet, F.A.J. 1989. *Int. J. Cancer* **43**: 115-1166; Ask, A., Persson, L. and Heby, O. 1992. *Cancer Lett.* **66**: 29-34; Seiler, N., Sarhan, S., Grauffel, C., Jones, R., Knödgen, B. and Moulinoux, J.-P. 1990. *Cancer Res.* **50**: 5077-5083; Persson, L., Holm, I., Ask, A. and Heby, O. 1988. *Cancer Res.* **48**: 4807-4811).

Depletion of intracellular polyamines in tumor cells is thus a well-known strategy in anticancer therapies. However, it is now of common knowledge that depleting

intracellular polyamines generally enhances polyamine uptake. To date, molecular information on the carrier molecules of the mammalian polyamine transport system is still unavailable. A few attempts have been made previously to design specific inhibitors of polyamine transport. Based on the finding that paraquat (4,4'-bipyridine) is a substrate of the putrescine transport system (Smith, L.L. and Wyatt, I. 1981. *Biochem. Pharmacol.* **20**, 1053-1058l; Rannels, D.E., Pegg, A.E., Clark, R.S. and Addison, J.L. 1985. *Am. J. Physiol.* **249**, E506-E513), a series of polypyridinium salts, including compounds with a low K_i against putrescine uptake and low acute toxicity for mammalian cells have been synthesized (Minchin, R.F., Martin, R.L., Summers, L.A. and Ilett, K.F. 1989. *Biochem. J.* **262**, 391-395). However, it is unclear whether such compounds can efficiently inhibit polyamine transport or are accumulated intracellularly. A number of polyamine analogs are effective competitors of polyamine uptake while being themselves substrates for transport (Seiler, N. and Dezeure, F., 1990. *Int. J. Biochem. Cell. Biol.* **27**: 425-442; Bergeron, R.J., and Seligsohn, H.W. (1986) *Bioinorg. Chem.* **14**: 345-355; Porter, C.W., Bergeron, R.J. and Stolowich, N.J. 1982. *Cancer Res.* **42**: 4072-4078; Porter, C.W., Basu, H.S., Feuerstein, B.G., Deen, D.F., Lubich, W.P., Bergeron, R.J., Samejima, K., and Marton, L.J. 1989. *Cancer Res.* **49**: 5591-5597; Pegg, A.E., Wechter, R., Pakala, R., and Bergeron, R.J. 1989. *J. Biol. Chem.* **264**: 11744-11749; Pegg, A.E., Nagarajan, S., Naficy, S. and Ganem, B. 1991. *Biochem. J.* **274**: 176-171; Porter, C.W., Ganis, B., Libby, P.R. and Bergeron, R.J. 1991. *Cancer Res.* **51**: 3715-3720).

More recently, a high-molecular weight (Mr=25Kd) spermine polymer has been described by Aziz *et al.* in USP 5,456,908, as a competitive inhibitor of polyamine transport, with a K_I in the 10^{-6}M range. In this patent document are disclosed two novel classes of polyamine transport inhibitors of high molecular weight, namely polymeric conjugates of normally transported substances (TS) of the structure $(TS)_m$ or conjugates of a polyamine and a protein or polypeptide (P) linked by known coupling agents and represented by (TS)-(P), wherein the repeating units of the polymer comprise the targeted polyamine. It is predictable that the inhibitors of Aziz *et al.* would be difficult to eliminate *in vivo* due to their high molecular weight and the high positive charge of the polymers, notwithstanding the risk of immunogenicity inherent to such high molecular weight inhibitors. The length of the polymers of Aziz *et al.* as well as their charge would cause

their adsorption to the cellular surface, which bears negative charges due to the presence of glycoproteins, e.g. sialic acid. Poly-L-Lysine, a commercially used compound analogous to high molecular weight polymers of polyamines by its positive charges, is known to promote a strong electrostatic interaction between the cell and its substrate, as in the induction of positive charges of gamma irradiation of synthetic polymers used to produce dishes for tissue culture. The polyamine transport inhibitors of Aziz *et al.* present the additional drawback of being highly cytotoxic. It is noteworthy that their spermine polymer is effective in decreasing contents of polyamines in cells even when not used in combination with DFMO and at concentrations much higher than those required to block polyamine uptake, which indicates inherent high toxicity of the compound toward the cell by a mechanism independent of polyamine transport *per se*. The cytotoxicity of the spermidine polymer of Aziz *et al.* is most probably explained by a non-specific effect on cellular physiology such as the cellular membrane. Although the authors pretend to demonstrate the specific action of the polymers with the fact that exogenous spermidine reverses the induced cytotoxicity, it is highly likely that competition between spermidine and the polymers or electrostatic interaction with the negatively-charged sites on the cellular membrane is responsible for the effect. The results obtained by Aziz *et al.* indicate that at least part of the effect observed with high molecular weight polymers is non-specific (Aziz, S.M., Tofiq, S.F., Gosland, M.P., Olson, J.W. and Gillespie, M.N. 1995. *J. Pharmacol. Exp. Ther.* **274**, 181-196). The usefulness of this spermine polymer for specifically blocking polyamine accumulation is therefore uncertain in view of its marked cytotoxicity.

Cysteamine and aliphatic monoamines of similar chain length such a n-butylamine and n-pentylamine have a low but significant ability to antagonize putrescine uptake (Gordonsmith, R.H., Brooke-Taylor, S., Smith, L.L. and Cohen, G.M. 1983. *Biochem. Pharmacol.* **32**, 431-437), although the mode of inhibition of these compounds has not been reported. The only other polyamine-like structure known to interact non-competitively with the polyamine transport system is pentamidine, an aromatic diamidine (Jones, H.E., Blundell, G.K., Wyatt, I., John, R.A., Farr, S.J. and Richards, R.J. 1992. *Biochem. Pharmacol.* **43**, 431-437), but the structural basis of its inhibitory activity is not yet clear.

It follows that there still exists a need for effective polyamine transport inhibitors which, while inhibiting the transport of polyamines, will not be internalized by the transport system and will not be toxic to the cell. The availability of low molecular weight inhibitors of polyamine transport would provide for the possibility of better renal elimination, as well as lower risks of being immunogenic. The availability of high-affinity, specific, but impermeant antagonists of polyamine transport would also allow to evaluate the antitumor efficacy of polyamine depletion strategies in vivo with minimal systemic cytotoxic effects.

There is much preclinical evidence supporting the hypothesis that the efficacy of the suicide inhibitor of ornithine decarboxylase, D,L-α-difluoromethylornithine (DFMO = Eflornithine) as a chemotherapeuticagent is limited by the enhanced ability of tumor cells to transport polyamines fromplasma sources. Plasma polyamines are partly derived from various dietary sources (7, 12, 18, 58-60, 62, 70) and from the activity of the gastrointestinalmicroflora, wihch produces and excretes very high amounts of putrescine and cadaverine (1, 17, 45, 50, 62, 70), which can enter the generalcirculation thorugh the enterohepatic pathway (6, 45). Other systemic contributions can also be attributed to polyamine excretion by peripheraltissues,including dying tumor cells (32, 35, 41, 42, 63, 64, 67, 79, 80). The enhanced uptake of polyamines by tumor cells results both from the increased polyamine transport activity that accompanies the malignant phenotype (11, 43, 51, 68, 69), and from the effect of DFMO itself, which causes a compensatory upregulation of polyamine uptake across the plasma membrane (9, 10, 14-16, 22, 25, 29, 31, 38, 39, 43, 47, 48, 50, 57, 61). One possible strategy that could be used to overcome this phenomenon would be to administer a pure antagonist of polyamine transport, i.e. a drug which binds with high affinity to the polyamine transporter, but which cannot be transported by this membranen protein. Unfotunately, no such compound is yet available, although some candidates have been tentatively proposed in the recent past (2, 3, 37).

SUMMARY OF THE INVENTION

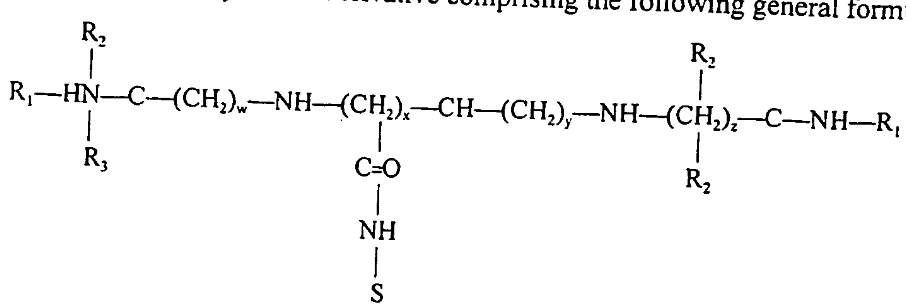
In accordance with the present invention, there is now provided polyamine transport inhibitors having a low molecular weight, less susceptible to immunogenicity and to non-specific interactions with the cellular membrane. These inhibitors have high affinity, are

specific, impermeant, pure antagonists of polyamine transport in mammalian cells while exhibiting minimal cytotoxic effects.

There is thus provided in accordance with the present invention synthetic derivatives of original polyamines, wherein the original polyamine is modified to comprise an amido group immediately linked to a carbon atom of said original polyamine, said synthetic derivatives inhibiting the cellular uptake of natural polyamines by specifically binding cellular transporters for said natural polyamines. Surprisingly, the immediate vicinity of the amido group to the backbone of the original polyamine preserves the specificity of the derivative towards the transporter while conferring thereto an impermeant character, providing a true antagonist. In a particularly preferred embodiment, the amido group is located between two internal nitrogen atoms of the original polyamine. In a most preferred embodiment, the synthetic derivative compromises a dimer wherein monomers of said dimer are linked together by a spacer side chain anchored to the amido group of each monomer.

Although natural polyamines such as putrescine, spermine and spermidine can be used as the original polyamine, other non natural polyamines can be used as a starting material for the making of synthetic derivatives as thought by the present invention.

Accordingly, a synthetic derivative comprising the following general formula



has been obtained, in which R_1 and R_1 independently represent a hydrogen atom or an alkyl group having 1 to 2 carbon atoms, R_2, R_2, or R_3 and R_3 independently represent a hydrogen atom or a methyl group, w and z independently represent an integrer of 2 or 3, x represents an integrer from 0 to n, n represents an integrer from 3 to 6, the sum of x and y equals n, and S represents a hydrogen atom or a molecule which cannot be captured by said natural polyamine transporter. The side chain S may be labeled and be used as a marker for a polyamine transporter. Furthermore, the side chain S can be varied to increase the affinity

of the derivative for the transporter. The side chain S may also become a spacer molecule useful in the formation of a dimer. This spacer side chain comprises a linear hydrocarbon-containing backbone of 3 to 8 atoms. The backbone may comprise sulfur, oxygen, or nitrogen atoms.

In a specific embodiment, the original polyamine is spermine. Three derivatives have been obtained therefrom: *N*-(2-mercaptoethyl)spermine-5-carboxamide (MESC), the disulfide from thereof, namely 2,2'-dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC), and *N*-[2,2'-Dithio(Ethyl, 1'-Aminoethyl)]spermine-5-carboxamide (DEASC).

It is another object of the invention to provide the use of all the above synthetic derivatives for inhibiting the activity of a natural polyamine transporter, comprising the step of contacting said transporter with an inhibitory effective amount of said synthetic derivative. This inhibition results in the control of the treatment of disorders involving unrestrained cell proliferation and/or differentiation where control of polyamine transport is required, when used in combination with an inhibitor of polyamine synthesis such as DFMO.

It is further another object of the invention to provide a use of the non-dimeric derivatives as a marker for a polyamine transporter, which comprises the steps of labeling said synthetic derivative, binding to said transporter said labeled synthetic derivative and detecting said bound labeled marker as an indication of the presence of said polyamine transporter. The above sequence of steps results in the diagnosis of a disorder involving unrestrained cell proliferation and/or differentiation where control of polyamine transport is required.

It is also another object of the invention to provide a pharmaceutical composition for treating disorders wherein control of polyamine transport is required, comprising anyone of all the above derivatives in adjunction with an acceptable pharmaceutical carrier. Preferably, this composition also comprises an inhibitor of polyamine synthesis, such as DFMO.

The applicants have unexpectedly discovered that the presence of a lateral amido group immediately linked to a carbon atom of the polyamine backbone of a synthetic derivative of an original polyamine confers impermeant properties to the so derived synthetic polyamine against the mammalian cell. It follows that the synthetic polyamine

derivatives of the present invention, by exhibiting high affinity for diamine and polyamine transport systems, block the transport of natural polyamines by competing therewith, while in the same time acting as poor substrate for intracellular uptake. The affinity of the polyamine derivative for the transporter system is further enhanced by increasing the length of a side chain anchored to the amido group of the derivative. The best affinity is achieved by dimerizing the polyamine derivative with the aid of a spacer molecule anchored at both ends to the amido group of each monomer. The flexibility of the chemical structure of the inhibitors of the present invention permits better optimization of the activity and affinity than a simple polymeric structure such as $(TS)_n$. For example, modifications to the polyamine backbone as taught by the present invention, such a methylation of C1 and C12, lowers the possibility of oxidation of the primary amides by the serum amine oxidase, which is present in mammalian sera. Additional modifications including adjunction to the lateral chain of alkylating groups that irreversibly modify residues that are essential to the activity of the polyamine transporter, such as carboxylic moieties of the carrier protein, are also contemplated in the present invention (Torossian, K., Audette, M., and Poulin R., 1996. *Biochem. J.* **319**: 21-28). The inhibitory action of the derivatives of the present invention is thus enhanced. By diminishing the amount of active transporters, additional modifications to the side chain that can be of potential therapeutic interest include the incorporation of reactive groups to the side chain that would allow the covalent modification of residues in the polyamine transporter by the principle of affinity labeling, and its subsequent irreversible inactivation.

This finding clearly demonstrates that modification of the chemical structure of the lateral chain optimizes the affinity of the polyamine derivative without augmenting to a great extent the molecular weight thereof. This markedly contrasts with the teachings of Aziz *et al.* who make use of high molecular weight polymers. Moreover, the mode of action of the inhibitors herein proposed, clearly different to that of Aziz *et al.* which relies upon their inherent cytotoxicity, is a competitive inhibition of the polyamine uptake.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only and with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

FIG 1 illustrates details of the synthesis of the compounds of the present invention, wherein a=triethylamine; b=di-*tert*-butyl dicarbonate; c=cyanuric chloride; d=cystamine dihydrochloride; e=3 N HCI; f=dithiothreitol: g=50 mM sodium phosphate in aqueous solution (pH=8.0); and wherein compound I is 5-carboxyspermine; compound II is tetra-Boc-5-carboxyspermine; compound III is 2,2^1-dithiobis[*N*-ethyl-(N^1, N^4, N^8, N^{12})-tetra-Boc-spermine-5-carboxamide; compound IV is *N*-[2,2^1-dithio(ethyl, 1^1-aminoethyl)]-N^1, N^4, N^8, N^{12}-tetra-Boc-spermine-5-carboxamide; compound V is 2,2^1-dithiobis(*N*-ethyl-spermine-5-carboxamide) octahydrochloride; compound VI is *N*-[2,2^1-dithio(ethyl, 1^1-aminoethyl)]-spermine-5-carboxamide (DEASC) and compound VII is *N*-(2-mercaptoethyl) spermine 5-carboxamide (MESC) tetrahydrochloride;

FIG 2 graphically illustrates the inhibition of [^{14}C]spermine transport by MESC, DESC and DEASC in human ZR-75-1 breast cancer cells. The rate of spermine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of DESC (O), MESC (●), and DEASC (□), using 1μM [^{14}C]spermine as substrate. Data are the mean ± SD of triplicate determinations;

FIG 3 graphically illustrates the inhibition of [^{3}H]spermidine uptake by spermine and DESC in ZR-75-1 cells. The rate of spermidine uptake was measured in ZR-75-1 cells grown as monolayers in 24-well culture plates in the presence of the indicated concentrations of spermine (O) and DESC (●) using 3μM [^{3}H]putrescine (A) or 1 μM [^{3}H]spermidine (B) as substrate. Data are the mean ± SD of triplicate determinations from a representative experiment;

FIG 4 illustrates graphically the Lineweaver-Burke analysis of putrescine transport inhibition by DESC and DEASC in ZR-75-1 cells. The rate of [^{3}H]putrescine uptake was determined in ZR-75-1 cell cultures with increasing concentrations of substrate (A) in the presence of 0 μM DESC (O), 3 μM DESC (●), 30 μM DESC (□) or 100 μM DESC (■) or (8) in the presence of 0 μM DESC (O), 20 μM DESC (●), 50 μM DESC (□) or 200 μM DESC (■);

FIG 5 illustrates graphically the structure of MESC thioether derivatives and their K_i values with respect to spermidine uptake in CHO-K1 cells. The various conjugates were prepared from MESC as described supra, and structure and name of the substituents are given in the first two columns from the left, wherein R corresponds to the group attached to sulfur in MESC (structure VII, Fig. 1). The rate of spermidine uptake was determined in CHO-K1 cells in the presence of increasing concentrations of the various MESC derivatives, using 1 μM [^{3}H]spermidine as substrate. K_i values are given as the mean ± SD of triplicate determinations from 2 to 3 experiments;

FIG 6 graphically represents the effect of DESC and MESC on the intracellular accumulation of [^{3}H]spermidine in ZR-75-1 cells, wherein at time 0 (A), 5 μM [^{3}H]spermidine was added to ZR-75-1 cell cultures grown in 24-well plates (1ml/well) in the presence of 200 μM MESC (●), 50 μM DESC (□) or 200 μM DESC (■), and accumulation of radiolabeled spermidine determined after the indicated interval. Control cells (O) received vehicle only. B, same as in A, except that 200 μM CHX was added at time 0 in the presence of 0 (●), 50 (□) or 200 μM DESC (■). Data are the mean ± SD of triplicate determinations;

FIG 7 illustrates the effect of spermine, MESC, DESC and DEASC on ZR-75-1 cell proliferations. Cells were incubated for 11 days in MEZR medium with the indicated concentration of spermine, DESC, MeSC, or DEASC in the presence (shaded bars) or absence (plain bars) of 1 mM of aminoguanidine, and DNA content per culture was then determined. Data represent the mean ± SD of triplicate determinations;

FIG 8 represents the effect of DESC on the reversal of DFMO-induced growth inhibition by exogenous spermidine in ZR-75-1 cells. Cells were incubated for 11 days in SD medium with the indicated concentrations of spermidine in the presence of 50 μM DESC (●), 1mM DFMO (□), or the combination thereof (■), or in the absence of drugs (O). Data are the mean ± SD of triplicate cultures;

FIG 9 represents the chromatographic profile of DESC and its degradation products in IMEM or PBS. DESC (50 μM) was added to 1ml of IMEM containing 10% fetal bovine serum in the absence (A) or presence (B) of 1mM aminoguanidine, or 1 ml PBS (C) in 24-well culture plates in the absence of cells. Media were analyzed after 20 minutes (solid lines) or 48 hours (dotted lines) of incubation at 37°C in 95% air: 5% CO_2, water-saturated

atmosphere for amine composition by ion-pair reversed-phase HPLC as described supra. Peaks 1 and 2 are degradation products of DESC, whereas peak 3 is a minor amount of DEASC initially present in the DESC preparation. Note the disappearance of peak 3 (DEASC) and the appearance of a shoulder (indicated by the arrow) at 42 minutes on the 48-hour profile in panel A; and

FIG 10 represents the time course of degradation of DESC in growth medium. At time 0, 50 μM DESC was added to 1ml of IMEM in 24-well culture plates and the content in DESC (○), compound 1 (Comp 1, ●) and compound 2 (Comp 2, □) determined by HPLC after the indicated incubation period at 37°C in a 5% CO_2 atmosphere. Data represent the mean of triplicate determinations from a representative experiment.

FIG 11. Structures of putrescine, of the natural polyamines spermidine and spermine, and of three cell-impermeant inhibitors of polyamine transport (DESC, DEASC and MESC).

FIG 12. Structure and scheme for the synthesis of unmethylated spermine analogs as polyamine transport inhibitors with a linker attached via amide bonds to the polyamine chains (BS-3, BS-4, BS-5 and BS-6 compounds). The method of synthesis is described in greater detail in Example 1.

FIG 13. Initial route of synthesis of terminal C-methylated, dimeric spermine analogs as transport inhibitors with a linker attached via an alkyl bond to the polyamine chains (BMS-3, BMS-4, BMS-5 and BMS-6). The steps presented in this figure describe the complete route of synthesis leading to the precursor N^1, N^4, N^8, N^{12}-tetra (Boc)-1, 12-dimethylspermine-5-carbinol (XV).

FIG 14. The first step in the coupling of N^1, N^4, N^8, N^{12}-tetra (Boc)-1, 12-dimethylspermine-5-carbinol (XV) to the linker L (= a *N*-mono-FMOC-diaminoalkane), toward the synthesis of BMS compounds.

FIG 15. The second step in the coupling of N^1, N^4, N^8, N^{12}-tetra (Boc)-1, 12-dimethylspermine-5-carbinol (XV) to the linker L (= a*N*-mono-FMOC-diaminoalkane) toward the synthesis of BMS compounds.

FIG 16. The final step of the synthesis of BMS compounds (XX); the Boc-protected, cross-linked 1, 12-dimethylspermine dimer is deprotected to generate the BMS compounds. BMS-3, BMS-4, BMS-5 and BMS-6 correspond to N^α, N^ω-bis ([1, 12-

dimethyl-spermine]-5-methyl)-diaminoalkanes where the diaminoalkane linker is 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, and 1,6-diaminohexane, respectively.

FIG 17A, 17B and 17C presents three classes of dimeric polyamine transport inhibitors according to the site of attachment of the linker (L) to the polyamine chain. FIG 17A Abbreviations used are R_1 = H, methyl, ethyl, or propyl; R_2 = H or methyl; $R_3 = Ch_2$, S, C=O or NH; $2< x <5$; $2 \leq y + \geq \leq 6$; L= a chemical structure (the linker) connecting covalently the two polyamine chains via alkyl, amide, ether or thioether bonds with a substituent group (R_3) attached on a carbon atom located between the two most internal amino groups of the polyamine chain. FIG 17B. Abreviations used are R_1 = H, methyl, ethyl, or propyl; R_2 = H or methyl; $2 < x < 5$; $2 < W < 8$; L' = a chemical structure (the linker) connecting covalently two polyamine chains via alkyl bonds with one of the two most internal amino groups of each polyamine chain. FIG 17C. Abbreviations used are R_1 = H, methyl, ethyl, or propyl; R_2 = H or methyl; $R_3 = Ch_2$, S, C=O or NH; $2< x <5$; $2 \leq y + \geq \leq 6$; $2 < W < 8$; L' - a chemical structure (the linker) connecting covalently two polyamine chains via an alkyl, amide, ether or thioether bond with a substituent group (R3) attached on one carbon atom located between the two most internal amino groups of one polyamine chain, to one of the two most internal amino groups of the other polyamine chain via an alkyl bond.

FIG 17A - C-linked dimeric analogs. R_1 is H, methyl, ethyl or propyl; R_2 is H or methyl; R_3 is an alkyl, amide, keto, ether, thioether, phosphono or sulfonyl group; x is greater than 2 and less than 5 ($2 < x < 5$), and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y + \geq \leq 6$). The linker L is any chemical structure covalently linked to the R_3 groups and which prevents the uptake of the analog.

FIG 17B - *N*-linked dimeric analogs. R_1 is H, methyl, ethyl or propyl; R_2 is H or methyl; x is greater than 2 and less than 5 ($2 < x < 5$), and w is greater than 2 and less than 7 ($2 < w < 7$). The linker L is any chemical structure covalently linked to one internal amino group of each polyamine chain and which prevents the uptake of the analog.

FIG 17C - C-linked/*N*-linked mixed dimeric analogs. R_1 is H, methyl, ethyl or propyl; R_2 is H or methyl; x is greater than 2 and less than 5 ($2 < x < 5$), the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y + \geq \leq 6$), and w is greater than

2 and less than 7 ($2 < w < 7$). The linker L is any chemical structure covalently linked to one internal amino group of one polyamine chain and to the R_3 of the other polyamine chain, and which prevents the uptake of the analog.

FIG 18. Initial route of synthesis of unmethylated, N^4-alkylated dimeric spermine analogs (FIG 17B). Steps leading to the synthesis of the intermediate N^1-benzyl, N^8, N^{12}-di(CBZ)-spermine.

FIG 19. Final steps for the synthesis of unmethylated, N^4-alkylated dimeric spermine analogs (FIG 17B, represented by type compound XXIX). For the aliphatic linker $-(CH_2)_n-$, $2 < n < 51$.

FIG 20. Initial route of synthesis of terminal C-methylated, N^4-alkylated dimeric spermine analogs (FIG 17B). Steps leading to the synthesis of the intermediate N^α, N^ω-bis (N-[N'-Boc-3-amino, 3-methylpropyl], N-[4-aminobutyl])-$\alpha\omega$-diminoalkane. For the alphatic linker-$(CH_2)_n$-, $2< n <51$.

FIG 21. Final steps for the synthesis of terminal C-methylated, N^4-alkylated dimeric spermine analogs (FIG 17B, represented by type compound XXXVIII). For the aliphatic linker-$(CH_2)_n$-, $2 < n < 51$.

FIG 22. Initial route of synthesis of 1,12-dimethylspermine dimers cross-linked through N^4-alkyl/5-alkyl attachments of the linker (FIG 17C). Steps leading to the synthesis of the intermediate N^α([N'-Boc-3-amino, 3-methylpropyl], N-[N'-FMOC-4-aminobutyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine)-methyl]-α, ω-diaminoalkane. For the aliphatic linker $-(CH_2)_n-$, $2 < n < 51$.

FIG 23. Intermediate route of synthesis of 1,12-dimethylspermine dimers cross-linked through N4-alkyl/5-alkyl attachments of the linker (FIG 17C). Steps leading to the synthesis of the intermediate N^α([N'-Boc-3-amino, 3-methylpropyl], N-[8-amino-5-aza-octanoyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine)-methyl]-α, ω-diaminoalkane. For the aliphatic linker $-(CH_2)_n-$, $2 < n < 51$.

FIG 24. Final route of synthesis of 1,12-dimothylspermine dimers cross linked through N4-alkyl/5-alkyl attachments of the linker (FIG 17C represented by type compound XLV). For the aliphatic linker $-(CH_2)_n-$, $2 < n < 51$.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Materials and Methods:

Sym-norspermidine, ornithine dihydrochloride and other reagents for organic syntheses were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO). Reversed phase silica gel liquid chromatography was performed with a Lichroprep™ RP-18 C_{18} silica gel column (40-63 μM ; BDH, St. Laurent, Qc., Canada) using a gradient of CH_3CN:MeOH:H_2O (25:35:40 to 50:30:20) as eluent. Homogeneity of synthetic products was assessed by thin-layer chromatography performed on 0.20 mm F_{254} silica gel 60 plates or 0.25 mm F_{254}S RP-18 reversed phase silica gel plates (E. Merck, Darnstadt, Germany). FIR spectra were obtained on a Perkin-Elmer 1600 spectrophotometer (FTIR series) and were expressed in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC/F 300 (300 MHz); ^{13}C were recorded at 75.47 MHz. Chemical shifts (δ in ppm) were referenced to $CDCl_3$ (7.26 ppm for ^1H and 77.00 ppm for ^{13}C). Mass spectra (MS) were recorded at the Mass Spectrometry Regional Center (University of Montreal, Montreal, Qc., Canada) by fast atomic bombardment mass spectrometry (FABMS) or liquid secondary ion mass spectrometry (LSMIS), using a VG AutoSpecQ™ and a Kratos MS50 TCTA, respectively.

[2,3-^{3}H(N]putrescine dihydrochloride (4.1 x 10^4 Cl/mol) and [1,8-^{3}H(*N*)]spermidine trihydrochloride (1.5 x 10^4 Cl/mol) were obtained from Dupont-New England Nuclear (Lachine, Qc., Canada). [5,8-^{14}C]spermine tetrahydrochloride (108 Cl/mol)) was purchased from Amersham (Arlington Heights, IL). DFMO was generously provided by the Marion Merrell Dow Research Institute (Cincinnati, OH). Fetal bovine serum (FBS) and Cosmic™ calf serum were from Hyclone (Logan, UT). The heterobifunctional reagent 1(-*p*-azidosalicylamido)-4-iodoacetamido)butane (ASIB) as obtained from Pierce (Rockford, IL). Lucifer Yellow (OY) iodoacetamide was purchased from Molecular Probes (Eugene, OR). Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, iodoacetamide, 5,5'-dithio(2-nitrobenzoic acid) and 3,4-diaminobenzoic acid as well as tissue culture reagents were purchased from Sigma. *Ortho*-phthaldialdehyde was purchased from Fluka (Ronkonkoma, NY) and other reagents for high-performance liquid chromatography (HPLC) were from Fisher Scientific (Montreal, Qc., Canada) or Aldrich (Milwaukee, WI).

Synthesis of 5-carboxyspermine (compound I)

Unless otherwise indicated, reactions were performed at room temperature. Compound I of Figure 1, namely 5-carboxyspermine, was synthesized using a known scheme (Behr, J.P. 1989. *J. Chem. Soc., Chem. Commun.* 101-103). Briefly to a stirred solution of 10.0 g (59.3 mmol) of ornithine hydrochloride dissolved in 250 ml MeOH were added 18.0 g (197 mmol) of tetramethylammonium hydroxide. After dissolution of ornithine salt, MeOH was evaporated, the mixture was then dissolved in 350 mL of dry dimethylformamide (HPLC grade; Aldrich, Milwaukee, WI) and the residual ammonium salt was filtrated, yielding ornithine as its free base. Following the addition of acrylonitrile (2.2. equivalents, 130.9 mmol), the mixture was stirred for 16 hours in the dark to give 10.6 g (yield=74%) of crude N^{α},N^{δ}-diethylcyanide ornithine, which was subsequently used without further purification. White solid: IR (film) v cm^{-1} 3372 (OH, acid), 2247 (CN); ^{1}H NMR δ ($CDCl_3$, 300 MHz) 1.48 (m, 4H, C<u>H</u>$_2$CHCOOH), 2.63 (m, 6H, 3 X C<u>H</u>$_2$N), 2.86 (2xt, J_1 =5.9 and J_2 -2.7 Hz, 4H, 2 X CH_2CN), 3.07 (t, J=7.2 Hz, 1H, C<u>H</u>COOH). To obtain 5-carboxyspermine KOH (2.7g, 48.0 mmol) was dissolved with vigorous stirring in 8 ml of 95% (v/v) Etoh and 10.5 g (44.1 mmol) of N^{α},N^{δ}-diethylcyanide ornithine were then added. The resulting mixture was placed under H_2 at 40 psi in a Burgess-Parr hydrogenator, using 2.09 g (24.4 mmol) or Raney nickel as catalyst (Behr, J.P. 1989. *J. Chem. Soc., Chem. Commun.* 101-103; Bergeron, R.J. and Garlich, J.R. 1984. *Synthesis*: 782-784). After 22 hours, Raney nickel was removed by filtration, and the solvent evaporated *in vacuo*, yielding 16.07 g of crude 5-carboxyspermine potassium salt. Yellow oil; IR (film) v cm^{-1} 3363 (OH, acid), 2937 (NH_2) no cyanide band; ^{1}H NMR δ ($CDCl_3$, 300 MHz) 1.53 (m, 2H, C<u>H</u>$_2$ NH_2), 2.65 (m, βH, C<u>H</u>$_2$NH), 3.09 (t, J=5.7 Hz, 1H, C<u>H</u>COOH).

Synthesis of 2,2'-Dithiobis(N-Ethyl-Spermine-5-Carboxamide) (DESC) and N-[2,2]-Dithio(Ethyl, 1'-Aminoethyl)] spermine-5-Carboxamide (DEASC)

Amine protection of 5-carboxyspermine by *tert*-butyl carbonyl (Boc) groups was performed as described (Ponnusamy, E., Fotadar, U., Spisni, A. and Fiat, D. 1986. *Syntheses*: 48-49). To 16.0 g (65.0 mmol) of crude 5-carboxyspermine potassium salt dissolved in 1.5 L MeOH were added 9.64 ml of 10% (v/v) triethylamine and 54.3 g (4.4 equivalents, 286 mmol) of di-*tert*-butyl dicarbonate. After stirring for 24 hours, solvent was evaporated, 100-150 ml H_2O were added and the resulting mixture was chilled at 0°C.

After adjusting pH at 2.2 with 2 N HCl, the Boc-product was extracted with ethyl acetate, dried over anhydrous $MgSO_4$ and purified by C_{18} reversed phase silica gel chromatography, yielding 3.3 g of pure tetra-Boc-5-carboxyspermine (Compound II, Fig. 1). Light yellow solid; IR (film) ν cm^{-1} 3356 (OH, acid), 1682 (C=O, amide); ^1H NMR δ ($CDCl_3$, 300 MHz) 1.32 (2 x s, 36 H, $(C\underline{H}_3)_3C$ from Boc-N), 1.90-1.40 (m, 9H, $C\underline{H}_2CH_2N$), 3.20-2.90 (m, 10H, $C\underline{H}_2N$); M (for $C_{31}H_{58}O_{10}N_4$) - 646.41;*m/z* (LSIMS) = 647.42 [(M+1)*]. Coupling of tetra-Boc-5-carboxyspermine (compound II) to cystamine was then performed in two steps based on the method of Venkataraman (Venkataraman, K. 1979. *Tetrahedr. Lett.* 32, 3037). To a solution of 1.15 g (1.78 mmol) of compound II in 20 ml dry acetone was added 0.27 mL (1.1 eq, 1.96 mmol) of triethylamine (freshly distilled on KOH) and 361 mg (1.1 eq, 1.96 mmol) of cyanuric chloride and the reaction mixture stirred overnight under N_2 to form the corresponding acid chloride. Cystamine dihydrochloride (241 mg; 1.07 mmol) was then suspended in dry triethylamine and added to the acid chloride form of compound II, with the resulting triethylamine concentration being at ≥4-fold excess relative to the latter. After stirring for 12 hours, the residual triazine oxide was filtrated, acetone was evaporated and the product extracted with $CHCl_3$, dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The crude compound was then purified by reversed-phase C_{18} column chromatography, yielding 0.682 g of 2,2'-dithiobis[*N*-ethyl-(N^1,*N4*,N^x,N^{12}-tetra-Boc-5-carboxamide (compound IV, Fig. 1). (m) Yellow oil; IR (film) ν cm^{-1} 1693 (C=O, amide); ^1H NMR δ ($CDCl^3$, 300 MHz) 1.38 (s, 36H, $(C\underline{H}_3)_3C$), 1.59 (m, 8H, $CH_2C\underline{H}_2CH^2$), 2.53 (6, *J*=5.7 Hz, 1H, $CON\underline{H}CH_2$), 2.73 (t, *J*=6.1Hz, 2H, $C\underline{H}_2S$),3.11 (m, 10H, $C\underline{H}_2NH$), 3.51 (m, 2H, $NC\underline{H}_2CH_2S$):M (for $C_{xx}H_{124}O_{18}N_{10}S_2$) = 1408.85; *m/z* (FABMS) = 1409.9 [(M+1)*].

Compound III (215 mg in MeOH) was then deprotected by addition of 1 ml of 3 N HCl, bringing the pH from 6.0 to =0.5. After stirring vigorously for 15 hours, the solvent was dried out *in vacuo* and the resulting compound purified by cation exchange chromatography with a Dowex 50W-X4 column (dry mesh; 100-200; Sigma) pre-equilibrated with H_2O and successively washed with H_2O, 1 N HCl, 2 N HCl, 4 N HCl and 6N HCl. Ninhydrin-positive fractions eluted with 6 N HCl were pooled and evaporated *in vacuo*, yielding 96 mg of pure 2,2'-dithiobis(*N*-ethyl-spermine-5-carboxamide)-octahydrochloride (DESC, Compound V, Fig. 1. White sold; mp 75-78°C; bp 118°C, ^1H NMR δ ($CDCl_3$, 300 MHz) 1.62 (m, 2H, $C\underline{H}_2CHCONH$), 1.97-1.80 (m, 6H, $C\underline{H}_2CH_2CH_2$),

2.74 (t, *J*=6.2 Hz, 2H, C<u>H</u>$_2$S), 2.92 (m, 10H, C<u>H</u> HN_2), 3.46 (dt, *J*=7.1 Hz, 2H, C<u>H</u>$_2$CH$_2$S),3.84 (t, *J*=7.0 Hz, 1H, C<u>H</u>CONH); M (for C H_{26} $O_{60}$$N_2$ S_{10}) = 608.96; *m/z* (FABMS)= 609.4 (M*).

Compound IV was similarly deprotected to yield *N*-[2,2'-dithio(ethyl, 1'-aminoethyl)]spermine-5-carboxamide (DEASC, Compound VI, Fig. 1). Yellow solid; mp 50-54°C; bp 109°C. 1H NMR δ ($CDCl_3$, 300 MHz) 1.89 (m,2H,C<u>H</u>$_2$ CHCONH), 2.10-2.29 (m, 6H C<u>H</u>$_2$CH$_2$CH$_2$), 3.04 (t-*J*=6.0 Hz, 2H, CONHC<u>H</u>$_2$CH$_2$S), 3.19 (t, *J*=7.4 Hz, 2H, SSCH$_2$C<u>H</u>$_2$HN$_2$), 3.25 (m, 10H, C<u>H</u>$_2$, NH), 3.51 (t, *J*=6.5 Hz, 2H, SSCH$_2$C<u>H</u>$_2$HN$_2$), 3.78 (m, 2H, CONHC<u>H</u>$_2$CH$_2$S), 4.11 (t, *J*=6.7 Hz, 1H, C<u>H</u>CONH). M (for $C_{15}H_{41}ON_5S_2$) = 380.62; *m/z* (LSIMS) = 381.24.

Synthesis of N-(2-Mercaptoethyl)spermine-5-Carboxamide [MESC]

DESC was dissolved in 50 mM sodium phosphate bugger, pH 8.0, containing 250 mM dithiothreitol (DTT), and incubated for 30 minutes at 37°C in a water bath. The mixture was then loaded on a Dowex™ 50W-X4 cation exchange column equilibrated with H_2O, and after washing with 5 column volumes each of 1 N HCl and 2 N HCl, the free thiol was eluted with 10 volumes of 4 N HCl, Amine-containing fractions, as identified by mixing 5 μl aliquots with 200 μl of an *o*-phthaldialdehyde solution (3.7 mM *o*-phthaldialdehyde; 0.4 M boric acid, pH 10.4; 1% v/v MsOH; 0.45% v/v 2-mercaptoethanol; 0.03% w/v Bri? 35) and hearing for 20 minutes at 37°C, were then pooled. The amount of *N*-(2-mercaptoethyl)-spermine-5-carboxamide [MESC] tetrahydrochloride (compound VII, Fig. 1) thus isolated was titrated for thiol equivalents with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (Jocelyn, P.C. 1987. *Meth. Enzymol.* 143, 44-67) using either cysteamine or DTT as standard. The yield of MESC using this procedure was virtually 100%, based on the number of thiol equivalents determined with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) and the expected number of thiol equivalents expected per mass of DESC. Finally, MESC purity was confirmed by ion-pair reversed-phase HPLC using post-column derivatization with *o*-phtaldialdehyde (Pegg, A.E., Wechter, R., Poulin, R., Woster, P.M. and Coward, J.K. 1989. *Biochemistry* 28: 8446-8453). [1]NMR δ ($CDCl_3$, 300 MHz) 1.91 (m, 2H, C<u>H</u>$_2$CHCONH), 2.08-2.24 (m, 6H, CH$_2$C<u>H</u>$_2$CH$_2$), 2.82 (t, *J*=6.3, 2H, CON

$HCH_2C\underline{H}_2SH$), 3.22 (m, 10H, $C\underline{H}_2NH$, 3.56 (m, 2H, $CONHC\underline{H}_2\ CH_2\ SH$), 4.11 (t, J=6.6, 1H, $C\underline{H}CONH$).

Synthesis of Thioether Adducts of MESC with Iodoacetamides

To 1 ml of an extemporaneously prepared, DTT-free solution of MESC (20 mM in H_2) were added 50 µl of 50 mM Tris-HCl (pH 7.0) and 105 µl of a 40 mM solution of either iodoacetamide, LY iodoacetamide or ASIB in a light-protected microcentrifuge tube, and the mixture was incubated for 2 hours at 37°C. The extent of thiol modification was assessed by measuring the amount of thiol remaining at the end of the incubation with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) as described above, and was determined to be essentially complete. Excess iodoacetamide was then inactivated by adding DTT to a final concentration of 40 mM and incubating the solution for 2 hours at 37°C. The resulting solutions of MESC adduct was used without further purification for [^{3}H]spermidine uptake assays conducted as described below. The effect of the respective DTT-inactivated iodoacetamide on spermidine transport was determined in parallel by incubating cells with the same reaction mixture from which MESC was omitted.

Cell Culture

Both ZR-75-1 human breast cancer cells and Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD). ZR-75-1 cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, 10 nM 17β-estradiol, and antibiotics [MEZR medium] (Huber, M. and Pouline, R. 1995. *Cancer Res.*, **55**, 934-943). CHO-K1 cells were routinely grown in α-Minimal Essential Medium supplemented with 10% Cosmic™ calf serum in a 5% CO_2 humid atmosphere at 37°C.

Even though the present invention has been described with a certain degree of particularity, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the following disclosure. Accordingly, it is intended that all such alternatives, modifications, and variations which fall within the spirit and the scope of the invention be embraced by the defined claims.

EXAMPLE 1
EFFECT OF INHIBITORS ON CELL PROLIFERATION

For growth studies, ZR-75-1 cells were cultured in MEZR medium or in phenol red-free RPMI 1640 supplemented with 2mM L-glutamine, 1 mM sodium pyruvate, 15 mM Hepes, antibiotics, 1 nM 17β-estradiol, 0.5 μg of bovine insulin per ml and 5% (v/v) charcoal-treated fetal bovine serum (SD medium), as indicated in the text. When polyamines or polyamine analogs were added to serum-containing media, 1 mM aminoguanidine was added to inhibit bovine serum amine oxidase (BSAO) activity (Morgan, D.M.L. 1989. in *The Physiology of Polyamines* (Bachrach, U., and Heimer, Y.M. eds) Vol. I, pp. 203-229, CRC Press, Boca Raton). The effect of the transport inhibitors on cell growth was measured by incubating ZR-75-1 cells for 11 days in medium supplemented with antagonist, polyamines and/or 1 mM DFMO as indicated, followed by calorimetric determination of DNA content with 3,4-d]aminobenzaic acid (Simard, J., Dauvois, S., Haagensen, D.E., Lévesque C., Mérand, Y. and Labrié, F. 1990. *Endocrinology* **126**: 3223-3231). Medium was changed every other day in these experiments because of the slow reaction of the compound with an unknown component present in the IMEM and RPMI 1640 medium formulation.

Polyamine Analysis

ZR-75-1 cells were plated in 100 mm culture dishes at 5 x 10^5 cells/dish in MEZR medium and grown for 5 days with medium changes every other day. Fresh MEZR medium containing the indicated concentration of transport antagonist was then added, plus or minus 200 μM cycloheximide (CHX), and cells were incubated for 1 or 6 hours. Medium was then removed, cell monolayers rinsed twice with 10 ml of ice-cold Ca^{2+}/MG^{2+}-free phosphate buffered-saline (PBS) (2.7 mM KCl; 1.5 mM KH_2PO_4; 8.1 mM Na_2HPPO_4; 137 mM NaCl), and harvested by centrifugation (2000 x g x 90 s at 4°C) following a 5 to 7 minute-incubation with bovine trypsin/EDTA solution (0.05%/0.02%, w/v) in Hanks' Balanced Salt Solution (Huber, M. and Poulin, R. 1995. *Cancer Res.* 55; 934-943). Cell pellets were resuspended in 300 μl of 10% (v/v) trichloroacetic acid or Tris-DTT buffer (50 mM Tris/HCl, 0.1 mM EDTA, 5 mM DTT, pH 7.5) and stored at -20°C until further analysis. For chromatographic analysis, samples were first quickly

thawed and incubated for 15 minutes at 37°C. Trichloroacetic acid was then added to DTT-containing samples to a final concentration of 10% (wt/v). Samples were dispersed for 2 minutes in a sonicating water bath, and pelleted in a microcentrifuge for 5 minutes. The trichloroacetic acid-insoluble pellet was solubilized in 300-500 μl of 1 N NaOH and used to determine protein content using bovine serum albumin (fraction V) as standard. Polyamine contents were then analyzed by ion pair reverse-phase HPLC with flurometric detection after postcolumn derivatization with *o*-phthaldialdehyde as described (Pegg, A.E., Wechter, R., Poulin, R., Waster, P.M., and Coward, J.K. 1989. *Biochemistry* **28**: 8446-8453; Huber, M., and Poulin, R. 1996. *Cancer Res.*, **55**: 934-943). In this system, putrescine, spermidine, spermine, DEASC and DESC were resolved with retention times of 18.5, 31.0, 35.0, 36.5, 37.5, and 44.0 minutes respectively.

DESC stability

DESC stability was tested by incubating the compound dissolved (at 50 μM) in PBS or in IMEM medium containing 10% (v/v) fetal bovine serum plus or minus 1 mM aminoguanidine in a humid 5% $_{CO2}$ atmosphere at 37°C and in the absence of cells. At indicated times, trichloroacetic acid was added to aliquots of this solution to a final concentration 10% (w/v) and the samples directly analyzed by HPLC as above.

Determination of Polyamine Uptake Activity

The rate of putrescine and spermidine transport was determined in ZR-75-1 cells incubated in serum-free RPMI 1640 medium as described (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R. 1995. *J. Biol. Chem.* **270**: 1685-1694), using [^{3}H]putrescine (30 Ci/mol) and [^{3}H]spermidine (20 Ci/mol), respectively as substrates for a 20 minute-assay period. Spermine uptake was similarly determined, using 1 μM [^{14}C]spermine (32 Ci/mol) as substrate. Uptake activity was expressed per amount of DNA as flurometrically determined using 3,4-diaminobenzoic acid (Simard, J., Dauvois, S., Haagensen, D.E., Lèvesque, C., Mèrand, Y. and Labrie, F. 1990. *Endocrinology*, **126**: 3223-3231). For the determination of spermidine uptake activity in CHO-K1 cells, 80% confluent cell monolayers were rinsed twice with PBS and incubated for 20 minutes at 37°C in 400 μl of buffer A (20 mM Tris-HCl, pH 7.4; 0.42 mM $CaCl_2$; 0.41 mM $MgSO_4$; 103 mM NaCL;

5.7 mM KCl; 1.1 mM D-glucose) containing 5 μM [^{3}H]spermidine (20 Ci/mol). Cell cultures were then washed twice with 1 ml PBS containing 5.7 mM *sym*-norspermidine. Cells were then lysed with 200-μl aliquot of 1 N NaOH and incubated for 30 minutes at 60°C. After neutralization with 200 μl of 1 N HCl, radioactivity was determined from a 250-μl of the cell lysate by scintillation counting. Uptake activity was expressed per amount of total cellular protein as determined by the method of Bradford (Bradford, M.M. 1976. *Anal. Biochem.* **72**: 248-254). Non-specific binding of radioactive substrate was similarly determined in parallel for both cell lines after a 15 second-incubation with 400 μl of ice-cold uptake solution.

Kinetic analyses

Kinetic analysis of polyamine transport was performed by determining uptake activity in the presence of a 3 μM [^{3}H]putrescine or 1 μM [^{3}H]spermidine plus increasing concentrations of nonradioactive substrate. $K_{??}$ K_i and V_{max} values were then estimated by Lineweaver-Burke analysis. For competitive inhibitors, K_i values were also estimated by measuring uptake activity in the presence of logarithmically increasing concentrations of antagonist, and using the Cheng-Prusoff equation (Cheng, Y.-C. and Prusoff, W.H. 1973. *Biochem. Pharmacol.* **22**: 3099-3108) by iterative curve fitting for a sigmoidal curve. For mixed competitive/noncompetitive inhibition, two methods were used to calculate kinetic constants. First, the equation

$$v = \frac{V_{max}}{\frac{K_m}{s}\left(1 + \frac{i}{K_i}\right) + \left(1 + \frac{i}{K_i'}\right)}$$

where *v*, *s*, and *i* are the transport velocity, substrate concentration and inhibitor concentration respectively, was used to calculate the inhibition constants for inhibitor/carrier complex formation (K_i) and carrier/inhibitor/substrate complex formation (K_i') (Dixon, M. and Webb, E.C. 1976. *Enzymes*, 3rd Ed., Academic Press, San Diego, CA). Alternatively, the value of K_i for a mixed competitor/non-competitor was estimated from the intersect of equations v^{-1} vs *i* at two different substrate concentrations (Dixon, M. and Webb, E.C. 1976. *Enzymes*, 3rd Ed., Academic Press, San Diego, CA).

Intracellular Accumulation

The time course of intracellular accumulation of spermidine in the presence of transport antagonists was determined by incubating ZR-75-1 cells in 24-well plates with DESC (50 or 200 μM) or MESC (200 μM) in dissolved in MEZR medium containing 5 μM [^{3}H]spermidine in the presence or absence of cycloheximide (CHX, 200 μM), and harvesting at the indicated times for the determination of intracellular radioactive contents, as described above for polyamine uptake assays.

Statistical Analysis

Statistical significance of differences between means was assessed by unpaired Student's *t*-tests. Unless otherwise indicated, results are expressed as means ± SD of determinations from triplicate cell cultures.

Design and Synthesis of DESC, DEASC and MESC

The original rationale for synthesizing MESC (Compound VII of Fig. 1) was to generate an affinity reagent with a thiol side chain that could be derivatized with fluorescent or radioactive sulfhydryl reagents to label the polyamine transporter. The precursor chosen for the synthesis, namely 5-carboxyspermine, has been previously used to prepare lipopolyamines for efficient DNA transfection (Behr, J.P. 1989. *J. Chem. Soc. Chem. Commun.* 101-103; Behr, J.P., Demeneix, B., Loeffler, J.-P. and Perez-Mutul, J. 1989. *Proc. Natl. Acad. Sci. USA* **86**: 6982-6986), and more recently, as a photoaffinity reagent to label the polyamine-binding site of casein kinase 2 (Leroy, D., Schmid, M., Behr, J.-P., Filhol, O., Pares, S., Garin, J., Bourgarit, J.-J., Chambaz, E.M. and Cochet, C. 1995 *J. Biol. Chem.* **270**: 17400-17406). The scheme used to prepare MESC involved the coupling of a cystamine bridge through amide bonds with two Boc-protected 5-carboxyspermine molecules to form DESC after removal of the Boc groups (Compound V of Fig. 1), followed by reduction of the DESC disulfide bridge. A small amount (10-15%) of the mixed MESC-cysteamine disulfide (DEASC, Compound VI; Fig. 1) was also generated in the coupling process. Complete separation of DEASC from DESC on a preparative basis proved to be difficult even using ion exchange chromatography (data not shown). Consequently, most DESC preparations contained a small amount (1-2%) of

DEASC after reversed-phase liquid chromatography on $C_{??}$ silica gel. DESC and DEASC were stable for months in aqueous solutions buffered at pH=7.0, whereas MESC solutions were supplemented with DTT to prevent oxidation.

Affinity of DESC, DEASC and MESC for the Mammalian Diamine and Polyamine Transport

In order to evaluate the suitability of the spermine conjugates as prospective affinity ligands, their relative ability to inhibit putrescine and polyamine uptake was evaluated. As shown in Fig. 2, DESC was the most potent antagonist of [^{14}C]spermine transport in ZR-75-1 cells, with a K_i value about 5-fold and 16-fold lower than that of DEASC and MESC, respectively. The ability of spermine to compete against [^{3}H]putrescine and [^{3}H]spermidine uptake was in fact only about 7-fold higher than that of DESC (Fig. 3). DESC (Fig. 4A) and MESC (data not shown) were pure competitive inhibitors of [^{3}H]putrescine uptake at concentrations up to 100 and 200 μM, respectively. On the other hand, inhibition of putrescine transport by DEASC belonged to a mixed competitive/non-competitive type (Fig. 4B). Table I summarizes the K_i values determined for DESC, MESC and DEASC toward putrescine, spermidine and/or spermine uptake, in relation with the mutual transport interactions between the latter substrates. Notably, K_i values of the three spermine conjugates with respect to putrescine uptake were 3-fold to 5-fold higher than for spermine uptake, unlike spermidine and spermine which both inhibited the uptake of either substrate with similar potency, and with a K_i roughly equal to their K_m as substrate.

TABLE I

K_I Values of Inhibition of Diamine and Polyamine Transport by MESC, DESC and DEASC in ZR-75-1 Cells

Compound	K_m or K_i (μ*M*)		
	Putrescine	Spermidine	Spermine
Putrescine	3.7 ± 0.4^{a}	125 ± 29^{a}	$0.23 \pm 0.13^{a,b}$
Spermidine	0.23 ± 0.05^{a}	0.49 ± 0.15^{a}	0.37 ± 0.09^{a}

Spermine	0.33 ± 0.02[a]	ND	0.20 ± 0.06[g]
DESC	1.6 ± 0.5[b]	2.7 ± 1.1[b]	5.0 ± 0.7[b]
MESC	22 ± 3[b]	ND	80 ± 31[b]
DEASC	5.3 ± 0.6 (K_i)[c]	ND	16 ± 3[d]
	4.1 ± 0.5 (K_i')		

Data annotated with *a* are from Lessard, M., Zhao, C., Singh, S.M., and Poulin, R. 1995. *J.Biol*, **270**: 1685-1694, *b* indicates data obtained with this work; mean ± SD of triplicate determinations from 2 to 4 different experiments; *c* corresponds to values of inhibition constants for carrier/inhibitor complex formation (K_i) and for carrier/inhibitor/putrescine complex formation (K_i') assuming a mixed competitive/non-competitive model; mean ± SD of triplicate determinations at 3 three inhibitor concentrations at two different substrate concentrations for a series of increasing inhibitor concentrations (Dixon, M, and Webb, E.C. 1976. *Enzymes*, 3rd Ed., Academic Press, San Diego, CA).

The relative potency of DESC and MESC as competitive inhibitors of polyamine uptake was also evaluated in CHO-K1 cells, in which they respectively exhibited K_i values of 0.92 ± 0.15 and 33.6 ± 7.2 μM (Fig. 5).

EXAMPLE 2
EFFECT OF SIDE CHAIN LENGTH AND SUBSTITUENTS ON SPERMIDINE TRANSPORT INHIBITION BY MESC DERIVATIVES

The observation that MESC was a less potent inhibitor of diamine and polyamine transport than DESC and DEASC suggested that the nature of the side chain strongly influences the interaction of these compounds with the carrier. The thiol side chain of MESC was thus derivatized with substituting groups of different sizes and charges through thioether linkage with three different iodoacetamides, namely LY iodoacetamide, ASIB and iodoacetamide itself, and the ability of the resulting complexes (MESC-LY, MESC-ASIB, and MESC-acetamide, respectively) to inhibit spermidine uptake was then evaluated. These studies were conducted using CHO-K1 cells.. As shown in Fig. 5, derivatization of the thiol group of MESC did not significantly (P>0.10) increase the K_i toward spermidine

uptake for the three conjugates studies. In the case of MESC-ASIB, K_i values might have been underestimated by partial inactivation of the polyamine carrier at the assay temperature, although the uptake reaction was conducted under subdued lighting. Thus, the results show that specific recognition of the spermine head of MESC can accommodate considerable variation in length, size, polarity or charge for the side chain without detrimental effect on its affinity for the polyamine carrier. Consequently, inhibitors having different side chains, while maintaining their inhibitory activity on polyamine transport are also encompassed by the present invention.

EXAMPLE 3
LACK OF PERMEATION OF DESC AND MESC THROUGH THE POLYAMINE TRANSPORT SYSTEM

The ability of ZR-75-1 cells to accumulate DESC and MESC was determined. Since DESC was eluted as a late, broad peak in the HPLC system used, DTT was added to cell extracts to reduce DESC to MESC and decrease the detection threshold. Results are shown in Table II. ZR-75-1 cells were incubated for 1 or 8 hours in MEZR medium in the presence of 50 or 200 μM DESC or MESC prior to determination of polyamine contents. CHX was added at 200 μM where indicated. Other details are provided under "Materials and Methods." Values are the mean ± SD of triplicate determinations from 2 independent experiments.

TABLE II
Intracellular Accumulation of DESC and MESC in ZR-75-1 Cells

Addition	Time (*h*)	Polyamine intracellular contents (*nmol/mg protein*)			
		Spermidine	Spermine	DESC	MESC
Control	1	0.69 ± 0.08	8.22 ± 0.48	-	-
	6	0.91 ± 0.07[a]	9.16 ± 0.13	-	-
+ 50 μM DESC	1	0.81 ± 0.14	8.27 ± 0.81	<0.01	<0.01
	6	0.73 ± 0.11	8.60 ± 0.29	<0.01	<0.01
+200 μM DESC	1	0.79 ± 0.11	8.77 ± 0.79	<0.01	<0.01
	6	0.76 ± 0.11	8.66 ± 0.26	0.12 ± 0.01	<0.01
+200 μM DESC + + CHX	1	0.75 ± 0.04	9.57 ± 0.31	<0.01	<0.01
	6	0.70 ± 0.03	9.55 ± 0.13	0.10 ± 0.01	<0.01
+ 50 μM MESC	1	0.95 ± 0.11	7.77 ± 0.06	<0.01	<0.01
	6	0.75 ± 0.11	8.13 ± 0.17	<0.01	<0.01
+ 200 μM MESC	1	1.15 ± 0.07[a]	8.93 ± 0.53	<0.01	0.020 ± 0.005
	6	0.81 ± 0.15	8.32 ± 0.43	<0.01	0.13 ± 0.06

[a] Significantly different (*P*<0.5) from control value at time = 1h. (?)

As shown in Table II, only trace amounts of DESC could be recovered in ZR-75-1 cells after a 6-hour incubation with 200 μM, but not with 50 μM; DESC could be detected only after reduction with DTT. These levels represent only about 1.5% of the accumulation measured in ZR-75-1 cells under identical conditions for spermine (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694). Moreover, inhibition of protein synthesis by cycloheximide (CHX), which is known to upregulate polyamine uptake by preventing the synthesis of a polyamine-induced feedback repressor of transport (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R. 1995. *J. Biol. Chem.* **270**: 1685-1694; Mitchell, J.L.A., Diveley, R.R., Jr. and Bareyal-Leyser, A. 1992. *Biochem. Biophys. Res. Commun.* **186**: 81-88), did not enhance DESC internalization, in marked contrast with its effect on spermidine accumulation under similar conditions (Fig. 6B) (Lessard, B., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694). Likewise, MESC was accumulated to measurable levels by ZR-75-1 cells only when present at 200 μM (cf.

Table II). Thus, neither DESC or MESC appear to be used as substrates for the polyamine transport system despite the high affinity of the former compound as an antagonist of diamine and polyamine uptake.

EXAMPLE 4
EFFECT OF DESC AND MESC ON INTRACELLULAR POLYAMINE ACCUMULATION

The time course of internalization of radiolabeled spermidine was determined in ZR-75-1 cells incubated for up to 6 hours in the presence of the impermeant agonists. As illustrated in Fig. 6A, steady-state [^{3}H]spermidine accumulation in the absence of competitor abruptly reached a near plateau after about 1 hour, which results from the induction of feedback inhibition of polyamine transport (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694). MESC and DESC decreased the initial rate of spermidine uptake according to their respective potency as competitive antagonists. Interestingly, spermidine accumulation in the presence of either inhibitor followed a pattern similar to that of control cells, i.e. a rapid phase during the first 60 minutes, followed by a much slower rate of accumulation thereafter, which was nearly independent of antagonist concentration. This pattern suggests that even cellular levels of newly internalized spermidine as low as 20% of those found under control conditions, e.g., in cells treated with 200 μM DESC, may induce a near maximal degree of feedback repression of polyamine transport. Nevertheless, even a 40-fold excess of the most potent antagonist (i.e. 200 μM DESC) only descreased net spermidine accumulation by only 50% after 6 hours. As previously observed ((Lessard, M., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694), CHX abolished the induction of feedback transport inhibition, resulting in a 4-fold increase in spermidine accumulation after 4 hours (Fig. 6B). Protein synthesis inhibition also enhanced spermidine accumulation in DESC-treated cells, a finding consistent with the onset of substantial feedback transport repression by subthreshold levels of internalized substrate. Thus, in the absence of the feedback mechanism, the highest concentration of DESC tested (200 μM) decreased net [^{3}H]spermidine accumulation by 80 to 85% after 6 hours and to a level lower than that found in control cells with a fully repressed uptake activity.

EXAMPLE 5
EFFECT OF DESC, DEASC AND MESC ON CELL PROLIFERATION

Due to the analogy of the novel transport antagonists with spermine, it might be surmised that they would exhibit significant cytotoxicity like the parent molecule. The marked toxicity of low ($<10^{-3}$M) spermine concentrations in biological media mostly results from catabolism by copper amine oxidases, which generates a dialdehyde, acrolein and H_2O_2 as deleterious products and can be irreversibly inhibited by carbonyl reagents such as aminoguanidine (Morgan, D.M.L. 1989. in *The Physiology of Polyamines* (Bachrach, U., and Heimer, Y.M. eds) Vol. I, pp. 203-229, CRC Press, Boca Raton). The biocompatibility of DESC, MESC, and DEASC was thus evaluated during a long-term (11-day) incubation with ZR-75-1 cells grown in RPMI 1640 containing 10% (v/v) FBS in the absence and presence of 1 mM aminoguanidine. As shown in Fig. 7, aminoguanidine alone had a slight inhibitory effect on ZR-75-1 cell growth as previously observed (Huber, M. and Poulin, R. 1995. *Cancer Res.* **55**: 934-943). Although DESC was only mildly growth inhibitory at 50 μM, there was an abrupt, aminoguanidine-resistant increase in toxicity at 200 μM. In contrast, spermine was acutely cytotoxic at 50 μM, an effect that was only partly prevented by aminoguanidine. MESC was considerably less toxic than its dimer, with a 35% decrease in cell growth at 200 μM which was not blocked by aminoguanidine. On the other hand, 50 μM DEASC caused a 20% inhibition of cell proliferation which could be completely prevented by the amine oxidase inhibitor. Thus, DESC, and to a much lesser degree, its thiol monomer MESC, are cytotoxic toward breast cancer cells at high concentrations through a mechanism that does not involve BSAO. Weak growth inhibition caused by the mixed MESC-cysteamine disulfide, however, apparently involved degradation by a copper amine oxidase.

EXAMPLE 6
EFFECT OF DESC ON RESCUE OF DFMO-INDUCED GROWTH INHIBITION BY EXOGENOUS SPERMIDINE

Although DESC is indeed a potent antagonist of polyamine accumulation, the slow residual uptake that occurred even at a 40-fold molar excess of inhibitor might be sufficient to counteract polyamine depletion by inhibitors of polyamine biosynthesis. This possibility

was assessed by comparing the ability of DESC to prevent the reversal of DFMO-induced growth inhibition by increasing concentrations of exogenous spermidine. At concentrations superior to 0.3 μM, spermidine inhibited ZR-75-1 cell proliferation by up to 20% (Fig. 8). This effect could be due to an incomplete inhibition of BSAO by aminoguanidine (Seiler, N. 1987. in *Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies* (McCann, P.P., Pegg, A.E. and Sjoerdsma, A. eds.), pp. 49-77, Academic Press, Orlando), since it was not observed in media supplemented with equine serum, which does not contain amine oxidase activity (Blaschko, H. and Hawes, R. 1959. *J. Physiol.* **145**: 124-131), instead of FBS (data not shown). The approximately 50% growth inhibition induced by 1 mM DFMO after an 11-day incubation was completely reversed by as little as 0.3 μM spermidine, whereas 0.1 μM spermidine already restored growth of DFMO-treated cells to 78% of control value. However, addition of 50 μM DESC was unable to prevent the reversal of DFMO-induced growth inhibition by spermidine, even at a DESC:spermidine ratio of 500. Essentially similar results were obtained using horse serum instead of FBS, or replacing RPMI 1640 medium, which contains 3.2 μM reduced glutathione that might undergo thiol/disulfide exchange with DESC, with thiol-free IMEM (data not shown).

EXAMPLE 7
STABILITY OF DESC IN BIOLOGICAL MEDIA

The inability of DESC to block the biological effect of exogenous spermidine, even when present at large molar excesses, might have been caused by its degradation in growth media. To assess this hypothesis, DESC solutions (20 μM) made in PBS or in sterile IMEM medium enriched with 10% (v/v) FBS were incubated for 20 minutes or 48 hours under cell-free conditions at 37°C in a humid 5% CO_2 atmosphere, and the polyamine analog was then analyzed by ion-pair reversed-phase HPLC. After 48 hours, degradation of DESC to two new amine-containing derivatives occurred in IMEM (Fig. 9A, B) but not in PBS (Fig. 9C), as evidenced by the appearance of a major (compound 1) and minor (compound 2) peaks of *o*-phtaldialdehyde-reactive material eluting earlier than DESC. Although aminoguanidine did not prevent DESC degradation to the two unknown products, it did prevent the degradation of a trace amount of DEASC (indicated as compound 3) initially present in the DESC preparations, thus confirming that DEASC can indeed be a

substrate of serum copper amine oxidase (Fig. 7). MESC could not be detected, indicating that DEC does not undergo reduction to MESC under conditions used for cell culture. Furthermore, the decomposition of DESC in IMEM showed an identical pattern in the presence or absence of FBS, which thus ruled out a serum component as being responsible for the degradation. Figure 10 shows that DESC was slowly degraded to compounds 1 and 2. After 48 hours, i.e. the interval at which freshly made DESC-containing media were added to cell cultures in growth experiments, 40% of the DESC originally present had been decomposed by IMEM. Identical results were obtained using RPMI 1640 medium instead of IMEM. Thus, the present inventors propose that a component present in IMEM and RPMI 1640 medium, but not in PBS, must be responsible for the degradation of DESC.

DESC, a novel type of spermine derivative, is shown to be endowed with high affinity for the polyamine transport system while being highly resistant to cellular uptake. The combination of these two attributes confers unique characteristics to DESC as a pure competitive antagonist of polyamine uptake.

As compared with spermine, the higher K_i of MESC against putrescine, spermidine and spermine uptake could owe to the presence of an amide linkage, which decreases the basicity of the neighboring secondary amino group of the spermine head ($pK_a = 5.5$ in comparison with 8.9-9.8 for spermine) (Tabor, C.W. and Tabor, H. 1984. *Ann. Rev. Biochem.* **53**: 749-790; Remy, J.-S., Kichler, A., Mordvinov, V., Schuber, F. and Behr, J.-P. 1995. *Proc. Natl. Acad. Sci. USA* **92**: 1744-1748, and/or may cause steric hindrance for its interaction with the polyamine binding site (Bergeron, R.J. and Seligsohn, H.W. 1986. *Bioinorg. Chem.* **14**: 345-355; Porter, C.W., Cavanaugh, P.F., Jr., Stolowich, N., Ganis, B., Kelly, E., and Bergeron, R.J. 1985. *Cancer Res.* **45**: 2050-2057). Despite the particular structural features of MESC as a ligand, its dimerization into DESC increased by up to 20-fold the affinity of the resulting structure for the polyamine transporter. There is no precedent for dimeric polyamine structures like DESC. Its overall design is reminiscent of that of 2-*N*-4-(1-azi-2,2,21,-tri-fluoroethyl)benzoyl-1,3-bis(D-mannos-4-yloxy)-2-propylamine, an impermeant ligand which binds to the exofacial domain of facilitative glucose transporters and bears two symmetrical sugar moieties linked tail to tail (Clark, A.E. and Holman, G.D. 1990. *Biochem. J.* **269**: 615-622). At least one mammalian glucose transporter, namely GLUT-1, exists as a tetrameric complex in its native form (Hebert,

D.N. and Carruthers, A. 1992. *J. Biol. Chem.* **267**: 23829-23838; Gould, G.W. and Holman, G.D. 1993, *Biochem. J.* **295**: 329-341). The stronger affinity of DESC relative to MESC could reflect a dyad symmetry in the organization of the transporter complex. Alternatively, dimerization of MESC into DESC could impose conformational constraints (e.g. due to electrostatic repulsion) that would favor recognition of the polyamine binding site of the carrier by each of the symmetrical spermine moieties.

MESC thioethers as diverse in size as MESC-LY, MESC-ASIB, or MESC-acetamide had K_i values virtually identical to that of MESC, indicating that the thiol group of MESC does not specifically determine its lower affinity as a polyamine transport inhibitor as compared with DESC. These data suggest that additional bulk on the side chain has little influence on the interaction of MESC with the polyamine transporter, in agreement with the observation that large substituents attached to the distal end of a spacer of sufficient length do not notably decrease the affinity of spermidine as a substrate for uptake (Holley, J.O., Mather, A., Wheelhouse, R.T., Cullis, P.M., Hartley, J.A., Bingham, J.P., and Cohen, G.M. 1992. *Cancer Res.*, **52**: 4190-4195). Unexpectedly, the MESC-cysteamine mixed disulfide (DEASC) was found to block putrescine uptake as a mixed competitor/non-competitor, whereas MESC and DESC behaved like pure competitive inhibitors of putrescine transport. Since the interaction of DESC or MESC with the polyamine transporter was strictly competitive, and because DEASC exhibits higher affinity than MESC as an inhibitor of diamine and polyamine transport, the spermine head and the cysteamine side chain of DEASC might be respectively responsible for the competitive and non-competitive components of its transport inhibition.

The biochemical properties of DESC clearly illustrate that the binding affinity of a compound can be dissociated from its ability to serve as a substrate for the polyamine transporter. The large size of DESC cannot be the main factor preventing its internalization through the channel-like portion of the transporter since MESC was also virtually impermeant. Thus, the mere attachment of an amido side chain on the spermine backbone would appear to be responsible *per se* for the impaired internalization of MESC and its derivatives. Indeed, N^4-alkylated spermidine derivatives are far better competitors of spermidine uptake than their N^4-acyl counterparts in mouse leukemia cells, in support of the notion that charged secondary amino groups are important in the interaction with the

polyamine carrier (Porter, C.W., Cavanaugh, P.F., Jr., Stolowich, N., Ganis, B., Kelly, E., and Bergeron, R.J. 1985. *Cancer Res.* **45**: 2050-2057). However, the latter argument cannot account for the fact that long-chain aliphatic α, ω-diamines with at least 6 to 7 methylene groups have an affinity comparable to that of spermidine (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694, Bergeron, R.J. and Seligson, H.W. 1986. *Bioinorg. Chem.* **14**: 345-355; Porter, C.W. and Bergeron, R.J. 1983. *Science* **219**: 1083-1085; Minchin, R.F., Martin, R.L., Summers, L.A. and Ilett, K.F., 1989. *Biochem. J.* **262**: 391-395; Gordonsmith, R.H., Brooke-Taylor, S., Smith, L.L. and Cohen, G.M. 1983. *Biochem. Pharmacol.* **32**: 3701-3709). A more likely explanation for the poor affinity of polyamines bearing an acyl side chain might be the steric hindrance due to the amide group, which restricts the freedom of rotation around the adjacent carbon and nitrogen atoms. There are indications that cyclic or pseudocyclic conformations of polyamines stabilized by hydrogen bonds might be energetically favored for recognition and/or internalization of substrates of the polyamine transport system (Lessard, M., Zhao, C., Singh, S.M. and Poulin, R., 1995. *J. Biol. Chem.* **270**: 1685-1694; Bergeron, R.J. and Seligsohn, H.W.1986. *Bioinorg. Chem.* **14**: 345-355). The formation of such folded conformers would be impaired by the presence of an amide group next to the polyamine chain. In support of this hypothesis, chlorambucil-spermidine, which bears a N-propyl chlorambucil carboxamide side chain on the central nitrogen of spermidine, is a good substrate of the polyamine transport system, with a K_m averaging that of spermidine (Holley, J.L., Mather, A., Wheelhouse, R.T, Cullis, P.M., Hartley, J.A., Bingham, J.P., and Cohen, G.M. 1992. *Cancer Res.* **52**: 4190-4195). In marked contrast, a spermidine conjugate with a chlorambucil carboxamide side chain directly attached at the C5 position of the spermidine head is a very poor substrate of the polyamine uptake system (Stark, P.A., Thrall, B.D., Meadows, G.G., and Abdel-Monam, M.M. 1992. *J. Med. Chem.* **35**: 4264-4269).

Although a 40-fold molar excess of DESC dramatically reduced the rate of spermidine uptake in ZR-75-1 cells, slow but continuous spermidine accumulation was still observed in the presence of the inhibitor. The low rate of polyamine internalization observed even in the presence of a large excess of DESC, in addition to the slow

decomposition of the inhibitor, may largely explain the complete inability of DESC to prevent polyamine-mediated prevention of growth inhibition by DFMO.

Since the affinity of MESC thioethers remains virtually unaffected relative to the unconjugated polyamine, MESC-ASIB might serve as a photoaffinity label to detect polyamine-binding proteins, including the polyamine carrier. Experiments are currently conducted with ^{125}I-labeled MESC-ASIB to assess its usefulness as a probe to identify the mammalian polyamine transporter. A recent report has described the specific labeling of discrete plasma membrane proteins using ^{125}I-labeled N^1-azidosalicylamido-norspermine and N^4-azido-salicylamidoethylspermidine as photoaffinity reagents (Felschow, D.M., MacDiarmid, J., Bardos, T., Wu, R., Woster, P.M. and Porter, C.W. 1995. *J. Biol. Chem.* **270**: 28705-28711). However, these conjugates are internalized by mammalian cells (Felschow, D.M., MacDiarmid, J., Bardos, T., Wu, R., Woster, P.M. and Porter, C.W. 1995. *J. Biol. Chem.* **270**: 28705-28711), and MESC-ASIB or similar derivatives could be useful as a photoactivatable probes to exclude labeling of intracellular proteins.

While not intending to be limited to any particular theory, the slow degradation of DESC observed in growth media, but not in PBS, was likely due to L-cystine, which is present at 100 and 200 μM in IMEM or RPMI 1640 medium, respectively, through the formation of mixed disulfides with DESC. Nevertheless, the cytotoxicity of high concentrations of DESC and MESC is unlikely to be solely due to the formation of such adducts, since MESC was less toxic than DESC, despite the fact that the free thiol group of the former would make it more reactive toward L-cystine]. The present data clearly show that DESC has remarkably low toxicity in comparison with its homolog spermine. Thus, the basic features of this molecule, including its resistance to BSAO, should be useful for the design of potent transport inhibitors with minor non-specific effects on cell viability. The inherent structural features of DESC that confer its high affinity and resistance to uptake should thus provide a useful framework for the design of potent irreversible inhibitors of polyamine transport, which could incorporate an alkylating group such as that used in the design of specific suicide substrates of mammalian glucose transporters (Clark, A.E., and Holman, G.D. 1990. *Biochem. J.* 269: 615-622; Lehmann, J., and Scheuring, M. 1995. *Carbohydrate Res.* 276: 57-74)].

Polyamine derivatives (natural or synthetic) comprising sulfur in the side chain have been made, because they conducted to the formation of dimers simply by forming a disulfide bridge. By-products which are not dimers have also shown an activity. However, it will be readily apparent to those skilled in the art that compounds being more stable than those containing sulfur atoms are contemplated. Therefore, the side chains used for increasing the affinity of the derivatives for a polyamine transporter and/or as substrates for labeling molecules and/or as a spacer in the making of a dimer can be varied to optimize the characteristics of the derivatives of the present invention.

Any equivalent structures or modifications obtainable without departing from the teachings and the spirit of this invention are considered as part of the scope thereof because the invention is in no way limited to the particularly disclosed embodiments, as reflected in the appended claims.

EXAMPLE 8

SYNTHESIS AND EVALUATION OF SPERMINE DIMERS AS INHIBITORS OF POLYAMINE TRANSPORT AND ENHANCERS OF EFLORNITHINE ACTION IN TUMOR CELLS AND TUMOR-BEARING ANIMALS

Novel spermine analogs will be synthesized and evaluated as blockers of transport in tumor cells simultaneously treated with D,L-α-difluoromethylornithine (FMO = Eflornithine). These molecules are based on the overall design of a prototype, 2, 2'-dithiobis(*N*-ethyl-spermine-5-carboxamide) (DESC). DESC has recently been reported to act as a competitive and potent antagonist of polyamine uptake in leukemia and breast cancer cells. DESC is proposed here to potentiate the chemotherapeutic efficacy of DFMO. While not intending to be limited to any particular theory, it is proposed that such effect is provided by preventing the replenishment of DFMO-treated tumor cells with polyamines from exogenous sources. Structural modifications to the molecule will improve it to a pharmacologically useful compound. These modifications include: [i] the replacement of the disulfide bridge with a fully reduced aliphatic chain to prevent its rapid reaction with biological thiols and disulfides and [ii] the addition of substituents to prevent its oxidative deamination by the ubiquitous plasma enzyme, serum amine oxidase.

Two types of DESC analogs will be synthesized, and characterized for their ability to inhibit polyamine transport and to enhance the therapeutic action of DFMO in various

tumor cell types, including animal models. The first type of analogs will be simply obtained by substituting the original cystamine side chain of DESC with α,ω-diamine cross-linkers of varying length. The synthesis of these analogs will help in the short term to optimize the length of the cross-linker chain, and to rapidly evaluate their relative ability to potentiate DFMO action *in vitro*. The second type of analogs will be made according to a new route of synthesis to introduce methyl groups at the extremities of the spermine-like backbone, and will also incorporate alkylation instead of acylation of the aliphatic, α,ω-diamine cross-linker in order to improve their affinity for the polyamine transport system, their potency as antagonists of uptake and as enhancers of DFMO therapeutic action. The pharmacological evaluation of the second-type analogs will be conducted in a standard mouse model bearing L1210 leukemia tumor cells treated with DFMO.

2,2'-dithiobis (*N*-ethyl-spermine-5-carboxamide) (DESC) and its thiol monomer, *N*-(2-mercaptoethyl) spermine 5-carboxamide (MESC) (Fig. 11) have been synthesized as precursors of photoaffinity labeling probes of polyamine-binding proteins (21). Characterization of the potency of DESC and MESC to inhibit polyamine transport unexpectedly showed that DESC has ≅20-fold higher affinity than MESC for the polyamine carrier. The marked difference in transport inhibitory potency between MESC and its dimer suggested that the carrier protein might have a dyadic symmetry, and that the conjugation of two spermine molecules through a cross-linking side chain could markedly enhance the interaction with the polyamine transporter. Moreover, neither DESC nor MESC is significantly internalized by human breast cancer cells or mouse leukemia cells at concentrations that saturate the polyamine carrier, indicating that they are essentially membrane-impermeant (21). The combination of high affinity and lack of carrier-mediated permeation of DESC provided the basis for a novel design of pure polyamine transport antagonists that could be used in combination with DFMO to enhance polyamine depletion in tumor cells exposed to physiological levels of exogenous polyamines.

DESC was designed for biochemical use. It was found to degraded in physiological media due to thiol-disulfide reaction with compounds such as L-cystine. DESC cannot efficiently counteract the ability of exogenous spermidine to reverse DFMO-induced cytostasis in breast cancer cells as a result of this instability (21). DESC is also subject to attack by serum amine oxidase (SAO), an ubiquitous plasma enzyme which oxidatively

deaminates aminopropyl groups, albeit to a much lesser degree than the parent compound, spermine. Modifications that further improve the design of DESC analogs that are part of particular embodiments of the present invention are:

(1) To use chemically inert, aliphatic diamines as cross-linkers to conjugate two spermine-like moieties; and/or

(2) To introduce methyl groups on the terminal carbons of the spermine-like backbones of the molecule. This latter modification will prevent/reduce the oxidation of spermine by SAO.

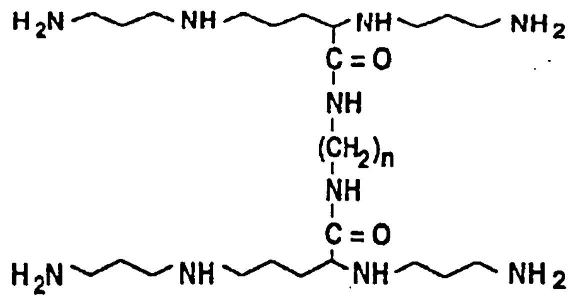
8.2) Synthesis and biochemical evaluation of unmethylated, stable DESC analogs

DESC analogs are prepared with unmodified spermine backbones but different side chain lengths as lead compounds to guide us in the design of methylated analogs described herein. This series of compounds will be synthesized in order to:

(i) Perform a structure-function study in the short-term to determine the optimal length of the cross-linker for inhibition of polyamine uptake.

Compounds VIIIa to VIIID (Fig. 12) will be rapidly available in amounts sufficient for *in vitro* testing. A refinement to the originally proposed route of synthesis will be the use of FMOC-blocked diamine precursors. One such diamine precursor is $NH_2(CH_2)_nNH_2$ where $n = 3$ to 6. Instead of simultaneously coupling two spermine-like moieties to a diamine cross-linker, each amino group of the diamine cross-linker was sequentially amidated to the spermine-like precursor with the *N*-FMOC-diamine, and then the other amine group was deprotected for the second amidation reaction. This sequential reaction scheme improves the purification of the spermine dimer from the spermine monoamide. This was difficult to achieve with the original method. The present method will improve the yield of desired product through a better control of the reaction stoichiometry.

The kinetic properties of these DESC analogs (abbreviated as BS-3, BS-4, BS-5 and BS-6; Fig. 12), as inhibitors of polyamine transport will be determined by uptake assays of radiolabeled putrescine, spermidine and spermine, according to procedures in Huber et al. (1996), J. Biol. Chem., 271: 27556-27563, which is specifically incorporated herein by reference. These structures are shown below.



wherein n is 3, 4, 5 or 6.

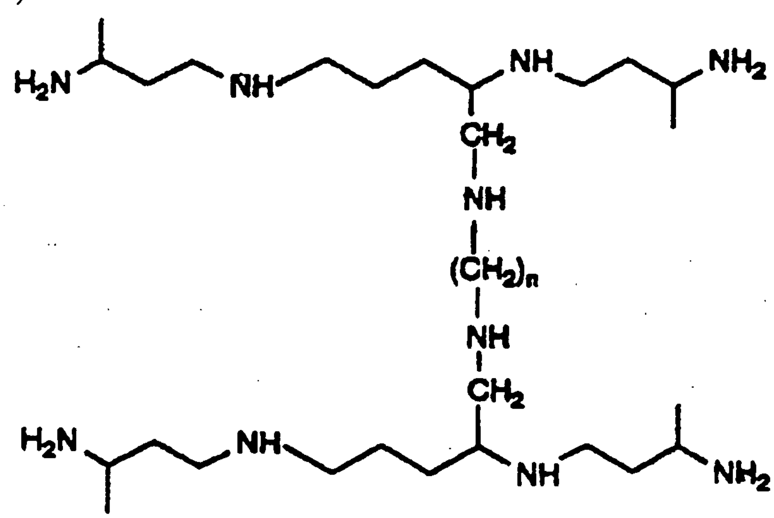
(ii) Use of these analogs to potentiate the effect of DFMO in the presence of exogenous polyamines, which is the main criterion of pharmacological activity for polyamine transport inhibitors.

These compounds are expected to be stable under cell culture conditions in the presence of aminoguanidine, a SAO inhibitor (13, 28, 40, 46, 49, 66, 67). These polyamine transport inhibitors will be evaluated using ZR-75-1 human breast cancer cells and L1210 mouse leukemia cells. Briefly, the rate of cell proliferation will be determined in ZR-75-1 and L1210 cells grown in the presence or absence of DFMO (1 and 5 mM, respectively), and of the transport inhibitor candidate to be analyzed, in the presence of increasing concentrations of putrescine or spermidine. The ability of the transport antagonist to prevent the reversal of DFMO-induced growth inhibition by exogenous putrescine or spermidine will provide a valid measurement of the pharmacological potential of these compounds as enhancers of DFMO action *in vivo*. These studies will also include (a) dose-response experiments to evaluate the cytotoxicity of these analogs and the optimal concentration for their use as inhibitors of polyamine uptake, and (b) measurement of the uptake of the transport inhibitors during incubation with tumor cells by HPLC, along with their effect on polyamine pools.

Since the latter type of inhibitors will rapidly provide the first stable DESC analogs available, the thorough analysis of their biological properties with cultured tumor cells will be important to validate the concept of spermine dimers as polyamine transport blockers. Moreover, the structure-function relationships of this series will help in refining the design of the methylated analogs described in the following section.

8.3) Design, synthesis and evaluation of oxidation-resistant, stable methylated DESC analogs

While replacing the nature of the cross-linking chain is a rather straightforward modification, the second alteration required considerable changes in the preparation scheme originally used for DESC synthesis. The reduction step proposed to obtain a 1,12-dimethylspermine 5-carboxyl methyl ester from a 1,4-bis(3-azidobutyramido) ornithine methyl ester (Fig. 13, XIII) could not spare the ester group, resulting instead into the formation of 1,12-dimethylspermine 5-carbinol (Fig. 3, XIV). Changing the nature of the ester group did not improve the synthesis since steric hindrance problems prevented amidation of the amino groups of ornithine methyl ester the most proximal to the ester group. The nature of the proposed precursor was modified, and two 1,12-dimethylspermine 5-methyl chains will be conjugated to an α,ω-diamine cross-linker through alkylation rather than through amide bonds (Figs. 14 to 16). This modification represents an improvement over the original design, since direct alkylation will lead to compounds with a higher affinity for the polyamine transporter - and higher potency as transport antagonists - as compared with the more rigid acylated analogs, as previously shown for spermidine analogs (8, 19, 44, 52, 54, 56). The proposed scheme of synthesis, for which steps IX to XIV have already been realized, is provided in Figs. 13 to 16. This improved scheme also includes the use of mono-FMOC-protected diamines as building blocks for cross-linking the dimethylspermine-5-methyl precursors, as described above for the unmethylated DESC analogs. The resulting compounds are abbreviated as BMS-3, BMS-4, BMS-5 and BMS-6 (Fig. 16; compounds XXa to XXd).



wherein n is 3, 4, 5 or 6.

The *in vitro* evaluation of this series will be conducted for the unmethylated DESC analogs. The effectiveness of a combination of polyamine depletion with the selected PA transport antagonist as an antitumor strategy will then be assessed *in vivo*. For this purpose, an established experimental cancer model, namely L1210 mouse leukemia, will be used to evaluate the therapeutic potential of the candidate transport inhibitor. This leukemia model is an aggressive tumor type with a median host survival time of 9 days in the absence of treatment. Moreover, it is completely resistant to DFMO as a single tumor agent *in vivo* (albeit very sensitive *in vitro*), whereas PA transport deficiency and/or reduction of exogenous PA sources confers a striking ability to DFMO to extend survival rates, with complete cure being observed in ≥75% of animals (1, 50).

<u>*Protocol 1*</u> - Toxicity will first be determined by single i.v. and i.p.injections of logarithmically increasing drug concentrations to mice and estimating the LD_{50}. Blood samples will be taken at intervals to measure the plasma drug concentration by ion pairing reverse-phase HPLC (22, 23). Body weight and liquid consumption will also be monitored for 10 days, at the end of which period animals will be sacrificed to evaluate the incidence of liver and kidney damage. A similar experiment will be conducted by dissolving the drug in the drinking water with free access to the animals.

<u>*Protocol 2*</u> - On day 0, mice will be injected with L1210 cells, with concomitant treatment with DFMO or vehicle, plus or minus 2 different sublethal doses of the transport antagonist on a daily schedule. Oral, i.v. and i.p. routes will be compared for the transport antagonist. Survival will be evaluated for up to 120 days, with regular body weight measurements and blood sampling to determine the steady-state plasma concentrations of inhibitor. L1210 cells are strongly immunogenic tumors and cured animals develop extended immunity against this leukemia (1). Thus, to evaluate the curative potential of the drug combination, survivors will be rechallenged with L1210 cells in the absence of treatment and survival monitored.

EXAMPLE 9

The present example demonstrates the utility of the present invention with the use of compounds that are analogs of spermine that include two chains connected to one another through a linker. The linker molecule that attaches the two spermine chains may be any spacer chain that is capable of bridging the polyamine chains.

The two chains may attach to the linker at an internal C atom or an N group within the chain. It is also possible for one chain to be connected to the linker through one of its carbon molecules, while the second chain attaches to the linker molecule through an N group within its chain.

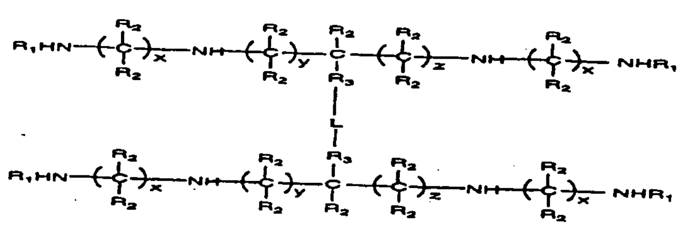
The general structure of compounds claimed include the following characteristics:

(1) The central carbon chain of the spermine backbone can have between 3 and 7 methylene groups or carbon atoms. This is the range of central chain length that can be accommodated with good affinity by the mammalian polyamine transporter (81).

(2) Each methylene group of the polyamine chains can be modified by methyl groups without compromising the ability of the inhibitor to interact with the polyamine transporter.

(3) The linkage between the polyamine chains and the spacer may comprise any type of linkage compatible with a $K_i \leq 20\mu M$ (relative to spermine) for the resulting inhibitor, such as direct alkyl substitution or ether group on the central methylene groups (Structure 1), or alkylation on the secondary amino (Structure 2) groups of the polyamine chain.

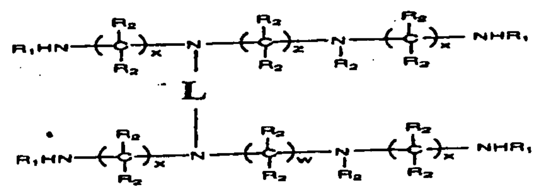
Structure 1



wherein R_1 is H, methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five ($2<x<5$), and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y+z \leq 6$). R_3 = Ch_2, S, C=O or NH; $2< x <5$; $2 \leq y + \geq \leq 6$; L= a chemical structure (the linker) connecting covalently the two polyamine chains via alkyl, amide, ether or

thioether bonds with a substituent group (R_3) attached on a carbon atom located between the two most internal amino groups of the polyamine chain.

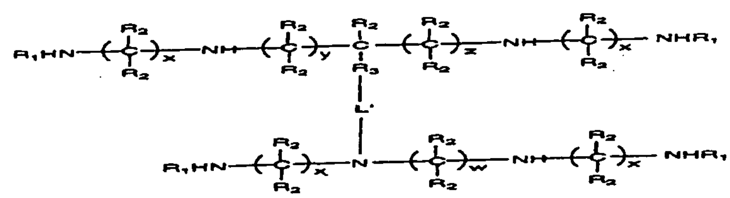
Structure 2



wherein R_1 is H, methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five ($2<x<5$), w is greater than 2 and less than 8 ($2<x<8$) and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y+z \leq 6$).

The invention when a carbon of one chain is attached by a linker to the nitrogen of a second chain is represented in Structure 3.

Structure 3



wherein R_1 is H, methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five ($2<x<5$), w is greater than 2 and less than 8 ($2<x<8$) and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y+z \leq 6$). R_3 = Ch_2, S, C=O or NH; $2< x <5$; $2 \leq y + \geq \leq 6$; L= a chemical structure (the linker) connecting covalently the two polyamine chains via alkyl, amide, ether or thioether bonds with a substituent group (R_3) attached on a carbon atom located between the two most internal amino groups of the polyamine chain.

Alkylation can be preferred over amidation because the former allows a greater flexibility to the polyamine chain to adopt the optimal conformation to interact with the polyamine transporter (81).

(4) The Linker (L) can be of any nature or chain length, as long as the total mass of the final structure does not exceed 3,000. These molecules may in other embodiments be described as having a total mass of between about 50 to about 2,5000, or about between 500 to about 1500 or about 1,000 as a total mass. By way of example, such linkers may comprise an alkyl, an ether, a thio ether, an amide, phosphono, keto, amine, and sulfonyl or a combination thereof.

The alkyl linker may comprise a carbon chain by a length of 2 to 50 carbons.

In some embodiments, the carbon chain will have a length of between 5 to about 25 carbons, or between 10 and 20 carbons, or in even other embodiments, the carbon length of 2 to about 15 or 12 carbons.

Synthesis of Embodiments

(1) **Unmethylated spermine analogs (FIG 17A)**: *N^1, N^4, N8, N^{12}*-tetra-Boc-5-carboxyspermine (IV, FIG 12) is first prepared as described (88). If the linker is going to be amidated to the polyamine chain, the carboxyl group used as an acceptor is activated with cyanuric chloride (88), and conjugated with a *N*-mono-FMOC diaminoalkane of the desired length to generate the corresponding *N^1, N^4, N8, N^{12}*-tetra-Boc-spermine-5-*N*-(*N'*-FMOC-aminoalkyl) carboxamide (V, FIG 12). The FMOC group of the latter compound is removed with 20% piperdine/DMF, and the resulting *N^1, N^4, N8, N^{12}*-tetra-Boc-spermine-5(*N*-ω-aminoalkyl) carboxamide (VI, FIG 12) is then reacted with the acid chloride form of *N^1, N^4, N8, N^{12}*-tetra-Boc-5-carboxyspermine (IV, FIG 12). The latter compound is then deprotected with HC1/CH_3COOH to obtain the corresponding N^α, N^ω-bis(spermine-5-oyl)-diaminoalkane, the desired transport inhibitor (VII, FIG 12). These compounds are symbolized as BS compounds, and BS-3, BS-4, BS-5 and BS-6 correspond to the forms where the diaminoalkane linker is 1,3-diaminopropane, 1, 4-diaminobutane, 1, 5-diaminopentane and 1, 6-diaminohexane, respectively. If the spacer is going to be alkylated to the polyamine chain, the carboxyl group used as an acceptor in an amidation reaction is first reduced to an alcohol with $LiAlH_4$. After protecting the amine groups with carbobenzoxy groups, the alcohol is then converted to a bromide with Pbr_3. The resulting CBX-protected spermine bromide is then reacted with a diamine spacer with a 2:1 stoichiometry to generate the CBZ-protected spermine dimer. This dimer is finally

deprotected by catalytic hydrogenation with Pd/C (82) to generate the unmethylated spermine dimer (the transport inhibitor). If an ether linkage is desired, the alcohol obtained as above is then converted to an alkoxide with sodium metal, and then reacted with an alkyl dihalide (e.g. 1,3-diiodopropane) with a 2"1 stoichiometry to generate the CBZ-protected polyamine dimer, which is then deprotected as above to generate the unprotected polyamine dimer (the transport inhibitor). As an alternative precursor to ornithine, one may use instead 2-hydroxyputrescine, synthesized as described (83), and proceed with carboxyethylation and catalytic hydrogenation as in the route using ornithine as precursor, to obtain 6-hydroxyspermine. The four amino groups of the latter are protected with carbobenzoxy groups, and the alcohol is converted into an alkyl halide or alkoxide for subsequent reaction with the spacer as described above.

(2) **Methylated spermine analogs (FIG 17A)**: For example, ornithine methylester (X, FIG 13) is synthesized as described (89) and is diamidated with two equivalents of 3-azidobutyric acid (XII, FIG 13) using DCC/OHB$_t$ (90) to generate N^1, N^4-bis (3-azidobutyryl)-ornithine methylester (XIII, FIG 13). The latter is then reduced using BH_3/THF (90) to obtain 1, 12-dimethylspermine-5 carbinol (XIV, FIG13). After protection of all four amino groups with Boc groups (XV, FIG 13), the carbinol group is activated with PBr_3 to generate 1, 12-dimethyl-N^1, N^4, N^8, N^{12}-tetra (Boc)-5-bromomethyl spermine (XVI, FIG 14), and reacted with FMOC-HN-$(CH_2)_n$NH2 (where $3 \le n \le 6$) to generate the corresponding 1, 12-dimethyl-N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine-5 (N^{α}-methyl, N^{ω}-FMOC-diaminoalkane) (XVII, FIG14). After removing the FMOC group with piperidine/dimethylformamide (XVIII,FIG 14), the free amino group of compound XVIII is alkylated with one equivalent of compound XVI to generate a N^{α}, N^{ω}-bis [1, 12-dimethyl-N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine]-5-methyl)-diaminoalkane (XIX, FIG 15). The latter compound is then deprotected with HCl/CH_3COOH to finally obtain the methylated spermine analog (BMS-3,BMS-4, BMS-5 and BMS-6), which are the desired transport inhibitors (XXa-d, FIG 15).

(3) **Unmethylated, *N*-alkylated spermine analogs (FIG 17B)**: A symmetrical dimer that can be made where the linker (L) bridges two polyamine derivative chains through one of the innermost, secondary nitrogens of each polyamine chain.

(a) *N*-benzyl-1,3-diaminopropane (XXI, FIG 18) is first obtained by catalytic hydrogenation of 3-(benzylamino)propiononitrile with Raney nickel as described (84).

(b) *N*-benzyl-1,3-diaminopropane is then *N*-alkylated with 3-bromobutyronitrile to generate N^1-benzyl, N^3-(3-cyanopropyl)-1,3-diaminopropane (XXII, FIG 18) (85).

(c) N^1-benzyl, N^3-(3-cyanopropyl)-1,3,-diaminopropane is protected with a Boc group (86) to generate N^1-benzyl, N^3-Boc, N^3-(3-cyanopropyl)-1,3,-diaminopropane (XXIII, FIG 18)

(d) N^1-benzyl, N^3-Boc, N^3-(3-cyanopropyl)-1,3,-diaminopropane is reduced to N^1-benzyl, N^4-Boc-spermidine (XXIV, FIG 18) by catalytic hydrogenation with Raney nickel (84).

(e) N^1-benzyl, N^4-Boc-spermidine is then cyanoethylated with acrylonitrile to generate N^1-benzyl, N^4-Boc, N^8-cyanoethyl- -spermidine, and reduced to N^1-benzyl, N^4-Boc-spermine by catalytic hydrogenation with Raney nickel (84) (XXV, FIG 18).

(f) The two free amino groups of N^1-benzyl, N^4-Boc-spermine are protected with CBZ groups as described (87) to generate N^1-benzyl, N^4-Boc, N^8, N^{12}-di(CBZ)-spermine (XXVI, FIG 18).

(g) N^1-benzyl, N^4-Boc, N^8, N^{12}-di(CBZ)-spermine is then deprotected to N^1-benzyl, N^8, N^{12}-di(CBZ)-spermine with trifluoroacetic acid as described (87) (XXVII, FIG 18).

(h) N^1-benzyl, N^8, N^{12}-di(CBZ)-spermine can then be cross-linked with an α,ω-dibromoalkane of the desired chain length to generate the corresponding bis(N^1-benzyl, N^8, N^{12}-di(CBZ)-spermine) dimer (XXVIII, FIG 19), which is then deprotected by catalytic hydrogenation with Pd/C (87) to generate the unmethylated, *N*-alkylated spermine dimer (the transport inhibitor) (XXIX, FIG 19).

(4) **Methylated, *N*-alkylated spermine analogs (FIG 17B):**

(a) The amino acid group of 3-aminobutyric acid is protected with Boc as described (89), and the resulting *N*-Boc-3-aminobutyric acid (XXX, FIG 20) is condensed with *N*-FMOC-1, 4-diaminobutane using DCC/OHBt (88) to obtain N^1-(*N*-Boc-3-aminobutyryl), N^4-FMOC-1, 4-diaminobutane (XXXI, FIG 20).

(b) N^1-(*N*-Boc-3-aminobutyryl), N^4-FMOC-1, 4-diaminobutane is then reduced to N^1-Boc-N^8-FMOC-1-methylspermidine with BH_3/THF (88) (XXXII, FIG 20).

(c) Two equivalents of N^1-Boc-N^8-FMOC-1-methylspermidine are then N^4-alkylated with one equivalent of the α, ω-diiodoalkane of the desired length to obtain the corresponding N^α, N^ω-bis (*N*-[*N*'Boc-3-amino, 3methylpropyl], *N*-[*N*'-FMOC-4-aminobutyl])-α, ω-diaminoalkane (XXXIII, FIG 20).

(d) The FMOC groups of the resulting N^α, N^ω-bis (*N*-[*N*'Boc-3-amino, 3-methylpropyl], *N*-[*N*'-FMOC-4-aminobutyl])-α,ω-diaminoalkane are then deprotected with 20% piperidine/DMF to yield the corresponding N^α, N^ω-bis (*N*-[*N*'Boc-3-amino,3-methylpropyl], *N*-[*N*'-4-aminobutyl])-α,ω-diaminoalkane (XXXIV, FIG 20), which is then condensed with 3-azidobutyric acid, prepared as described (88), using DCC/OHBt, to generate the corresponding N^α, N^ω-bis(*N*-[*N*'Boc-3-amino,3-methylpropyl],*N*-[*N*'-8-amino-5-aza-octanoyl])-α,ω-diaminoalkane.

(e) The N^α,N^ω-bis(*N*-[*N*-Boc-3-amino,3-methylpropyl],*N*-[*N*-8-amino-5-aza-octanoyl])-α, ω-diaminoalkane is then reduced to the corresponding N^α, N^ω-bis(*N*-[*N*-Boc-3-amino,3-methylpropyl], *N*-[*N*-8-amino-5-aza-octyl])-α,ω-diaminoalkane with BH_3/THF (88) (XXXVII, FIG 21).

(f) The Boc groups of N^α, N^ω-bis (*N*-[*N*-Boc-3-amino, 3-methylpropyl], *N*-[*N*-8-amino-5-aza-octyl])-α,ω-diaminoalkane are then removed with HC1/CH_3COOH to generate the desired transport inhibitor, a N^α, N^ω-bis (*N*-[3-amino, 3-methyl-propyl], *N*-[8-amino-5-aza-octyl])-α, ω-diaminoalkane (XXXVIII, FIG 21).

(5) **1,12-Dimethylated spermine dimers cross-linked through *N*-alkyl/C-alkyl attachments of the linker (FIG 17C):**

Dimeric polyamine transport inhibitors of a different type can be generated by cross-linking one polyamine chain to a linker through a *N*-alkyl bond as in Examples 3 and 4 above, and the other polyamine chain via a C-linked anchor lying between the two innermost secondary amino groups as in Examples 1 and 2 above. Such compounds (as terminal C-methylated spermine analogs) can be obtained as follows:

(a) N^1-Boc-N^8-FMOC-1-methylspermidine (XXXII, FIG 20), obtained as described above (Example 4, steps a to b), is N^4-alkylated using an ω-bromoalkylphthalimide of the desired length as described (92), to generate the corresponding N^1-Boc, N-alkylphthalimide, N-FMOC-1-methylspermidine (XXXIX, FIG 22).

(b) The phthalimide group of N^1-Boc, N^4-alkylphthalimide, N^8-FMOC-1-methylspermidine is removed with hydrazine in EtOH (88, 90) to generate the corresponding N^1-Boc, N^4-(ω-aminoalkyl), N^8-FMOC-1-methylspermidine (XL, FIG 22).

(c) The free amino group of N^1Boc, N^4-(ω-aminoalkyl), N^8-FMOC-1-methylspermidine is then alkylated with N^1, N^4, N^8, N^{12}-tetra (Boc)-1, 12-dimethyl-5-bromomethylspermine prepared as described in Example 2 (XVI, FIG 14) to obtain the corresponding N^α([N'-Boc-3-amino, 3-methylpropyl], N-[N'-FMOC-4-aminobutyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra(Boc)-spermine)-methyl]α,ω-diaminoalkane (XLI, FIG 22).

(d) The FMOC group of N^α([N'-Boc-3-amino, 3-methylpropyl], N-[N'-FMOC-4-aminobutyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine)-methyl]α,ω-diaminoalkane is then removed with 20% piperidine/DMF, and the resulting N^α([N'-Boc-3-amino, 3-methylpropyl], N-[4-aminobutyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine)-methyl]α, ω-diaminoalkane (XLII, FIG 23) is condensed with 3-azidobutyric acid (XXX, FIG 21) as in Example 4 (step d) above, to generate the corresponding N^α([N-N'-Boc-3-amino, 3-methylpropyl], N-[8-amino-5-aza-octanoyl]), N^ω-[5-(N^1, N^4, N^8, N^{12}-tetra (Boc)-spermine)-methyl]α, ω-diaminoalkane (XLIII, FIG 23).

(e) N^{α}(N-[N'-Boc-3-methylpropyl],N-[8-amino-5-aza-octanoyl]),N^{ω}-[5-(N^{1}, N^{4}, N^{8}, N^{12}-tetra (Boc)-spermine)-methyl]-α, ω-diaminoalkane is then reduced to N^{α}(N-[N'-Boc-3-amino, 3-methylpropyl], N-[8-amino-5-aza-octyl]), N^{ω}-[a5-(N^{1}, N^{4}, N^{8}, N^{12}-tetra (Boc)-spermine)-methyl]-α, ω-diaminoalkane (XLIV, FIG 24) with BH_3/THF as in Example 4 (step e) above, and the Boc groups of the resulting compound are removed with HC1/CH3COOH to generate the corresponding N^{α}(N-[N'-[3-amino, 3-methyl-propyl], N-[8-amino-5-aza-octyl]), N^{ω}-[5-(1, 12-dimethyl-spermine)-methyl]-α,ω-diaminoalkane (XLV, FIG 24), which is the desired polyamine transport inhibitor.

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CLAIMS

WHAT IS CLAIMED IS:

1. A synthetic derivative of an original polyamine, wherein a carbon atom of said original polyamine comprises an amide group, said synthetic derivative inhibiting the cellular uptake of a natural polyamine by specifically binding a cellular transporter for said natural polyamine.

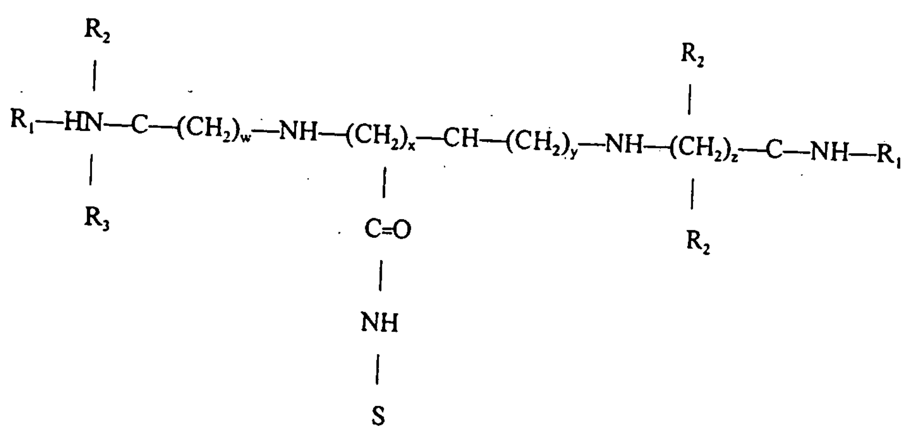
2. A synthetic derivative according to claim 1, wherein the carbon to which said amido group is located is between two internal nitrogen atoms of said original polyamine.

3. A synthetic derivative according to claim 2 which comprises a dimer of said original polyamine, the monomers of said dimer being liked together by a spacer side chain anchored to the amido group of each monomer.

4. A synthetic derivative according to claim 3, wherein the original polyamine is selected from the group consisting of putrescine, spermidine and spermine.

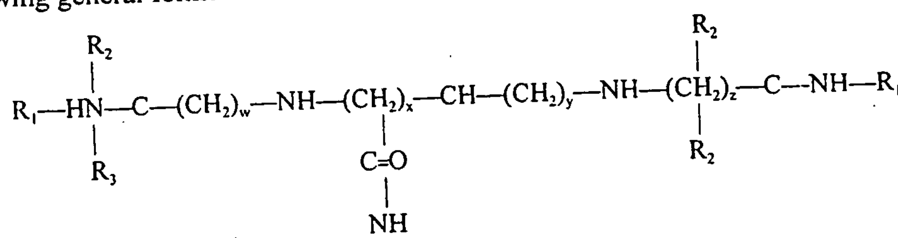
5. A synthetic derivative according to claim 4, wherein the original polyamine is spermine.

6. A synthetic derivative according to claim 2, wherein said synthetic derivative has the following general formula:



in which R_1 and R^I_1 independently represent a hydrogen atom or an alkyl group having 1 to 2 carbon atoms, R_2, R^I_2, or R_3 and R^I_3 independently represent a hydrogen atom or a methyl group, w and z independently represent an integrer of 2 or 3, x represents an integer from 0 to n, n represents an integer from 3 to 6, the sum of x and y equals n, and S represents a hydrogen atom or a molecule which cannot be captured by said natural polyamine transporter.

7. A synthetic derivative according to claim 3, wherein said monomer has the following general formula:



in which R_1 and R^I_1 independently represent a hydrogen atom or an alkyl group having 1 to 2 carbon atoms, R_2, R_2, or R_3 and R_3 independently represent a hydrogen atom or a methyl group, w and z independently represent an integrer of 2 or 3, x represents an integrer from 0 to n, n represents an integrer from 3 to 6, the sum of x and y equals n, and wherein the spacer side chain comprises a linear hydrocarbon-containing backbone of 3 to 8 atoms.

8. A derivative according to claim 7, wherein said backbone comprises sulfur, oxygen or nitrogen.

9. A derivative according to claim 8, wherein w=2, z=2 x=0 and y=3.

10. A derivative according to claim 7, wherein w=2, z=2, x=0 and y=3

11. A derivative according to claim 8, wherein w=2, z=2, x=0 and y=4

12. A derivative according to claim 11, wherein the hydrocarbon-containing backbone comprises a disulfide bridge.

13. A derivative according to claim 9, which is *N*(2-mercaptoethyl)spermine-5-carboxamide.

14. A derivative according to claim 9, which is *N*(2,2'-dithio(ethyl, 1'-aminoethyl)spermine-5-carboxaimide.

15. A derivative according to claim 12 which is 2,2'-dithiobis(*N*-ethylspermine-5-carboxamide).

16. The use of a synthetic derivative according to any one of claims 1 to 15 for inhibiting the activity of a natural polyamine transporter comprising the step of contacting said transporter with an inhibitory effective amount of said synthetic derivative.

17. The use according to claim 16, which results in the control or the treatment of disorders involving unrestrained cell proliferation and/or differentiation where control of polyamine transport is required, when used in combination with an inhibitor of polyamine synthesis.

18. The use according to claim 16 wherein the inhibitor of a polyamine synthesis is DFMO.

19. The use of the synthetic derivative of any one of claims 1, 2, 6, 9, 13 and 14 as a marker for a polyamine transporter, wherein said synthetic derivative comprises a detectable label having affinity for a polyamine transporter.use comprises the steps of labeling said synthetic derivative to provide a labeled synthetic derivative, binding said labeled synthetic derivative to a polyamine transporter, and employing said labeled synthetic derivative bond to a polyamine transporter as a marker for the detection of a polyamine transporter.

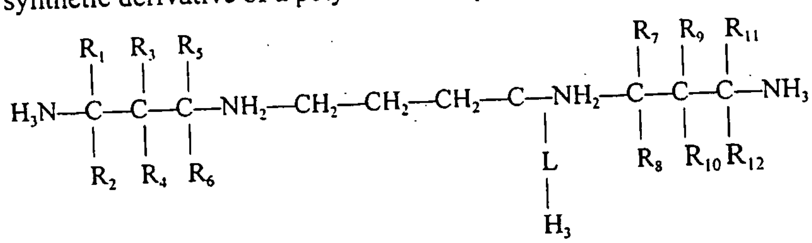
20. The use according to claim 19 which results in the diagnosis of a disorder involving unrestrained cell proliferation and/or differentiation where control of polyamine transport is required.

21. A pharmaceutical composition for treating disorders wherein control of polyamine transport is required, comprising a synthetic derivative according to any one of claims 1 to 15 in combination with an acceptable pharmaceutical carrier.

22. A pharmaceutical composition according to claim 21, which further comprises an inhibitor of polyamine synthesis.

23. A pharmaceutical composition according to claim 22, wherein said inhibitor of polyamine synthesis is a α-difluoromethylornithine.

24. A synthetic derivative of a polyamine comprising

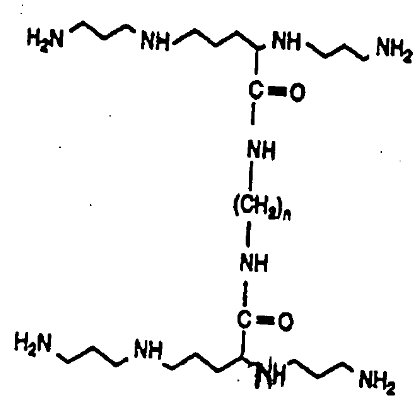


wherein R^1 or R^2 and R^{11} or R^{12} is methyl, R^3 through R^{10} is H or methyl, and L is a linker comprising a chemical entity covalently attached to a polyamine chain capable of modifying the membrane permeability of a polyamine analog.

25. The derivative of claim 24 wherein the L is a α,ω-diamine cross-linker.

26. The synthetic derivative of Claim 25 wherein at least one of R_1-R_{12} are methyl groups.

27. A synthetic derivative of a polyamine comprising a BS compound having a structure:



wherein n is 3, 4, 5 or 6.

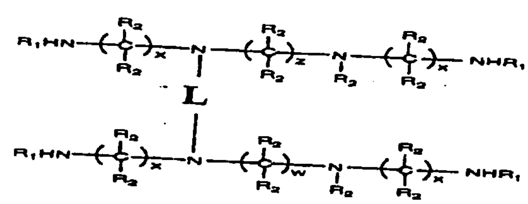
28. The synthetic derivative of claim 27 wherein n is 3.

29. The synthetic derivative of claim 27 wherein n is 4.

30. The synthetic derivative of claim 27 wherein n is 5.

31. The synthetic derivative of claim 27 wherein n is 6.

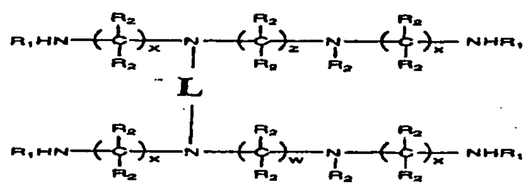
32. A synthetic derivative of a polyamine comprising Structure 1:



wherein R_1 is H, methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five (2<x<5), w is greater than 2 and less than 8 (2<x<8), and R_3 is an alkyl, amide, keto, ether, thioether, phosphono or sulfonyl group; x is greater than 2 and less than 5 (2 < x < 5), and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq$ $y + \geq \leq 6$)

33. The synthetic derivative of claim 32 wherein x is 3, R_1 is hydrogen, R_2 is a methyl (Ch_3-) group for the carbon atom located at each extremity of the two polyamine chains connected by the linker L, and is a hydrogen atom for all other carbons, y + Z = 3, and L is $-CH_2-HN(CH_2)_nNH-CH_2-$, where n = 3, 4, 5 or 6.

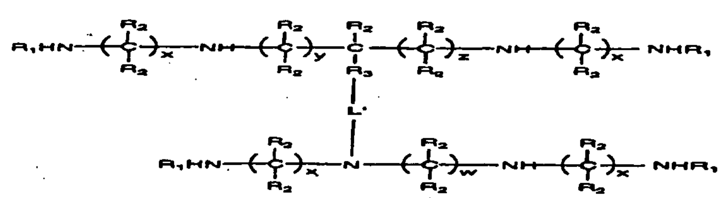
34. A synthetic derivative of a polyamine comprising Structure 2:



wherein R_1 is H, methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five ($2<x<5$), w is greater than 2 and less than 8 ($2<x<8$) and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y+z \leq 6$).

35. The synthetic derivative of claim 34 wherein x=3, R_1 is a hydrogen atom, R_2 is a methyl (CH_3-) group for the carbon atom located at each extremity of the two polyamine chains connected by the linker L, and is a hydrogen atom for all other carbons and w = 4.

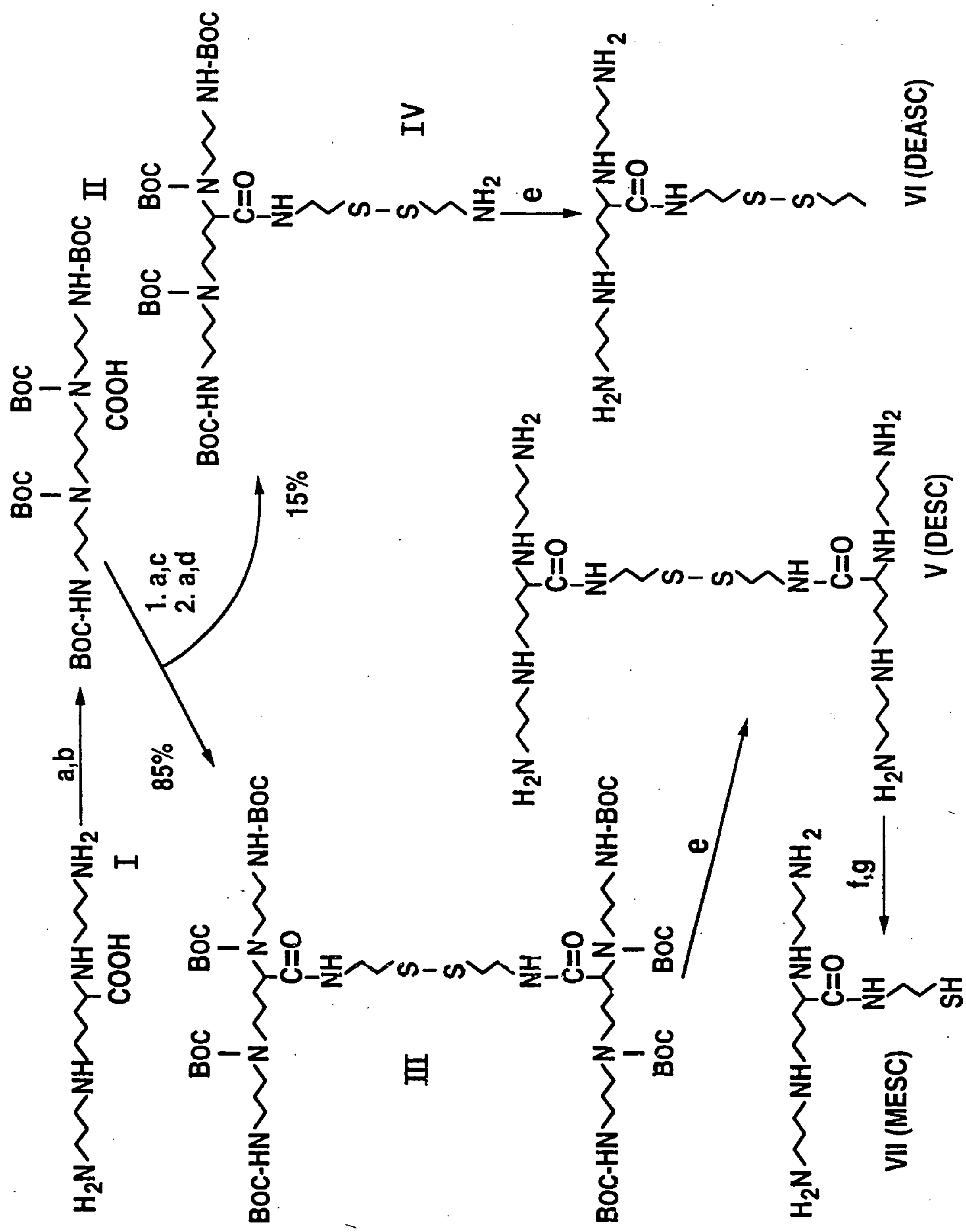
36. A synthetic derivative of a polyamine comprising Structure 3:



wherein R_1 is H. methyl, ethyl or propyl, R_2 is H or methyl, x is greater than two and less than five ($2<x<5$), w is greater than 2 and less than 8 ($2<x<8$), and R_3 is an alkyl, amide, keto, ether, thioether, phosphono or sulfonyl group; x is greater than 2 and less than 5 ($2 < x < 5$), and the sum of y+z is greater than or equal to 2 and less than or equal to 6 ($2 \leq y + \geq \leq 6$).

37. The synthetic derivative of claim 35 wherein L is an alkylbeing a carbon length of 12 to about 14 carbons.

Fig. 1



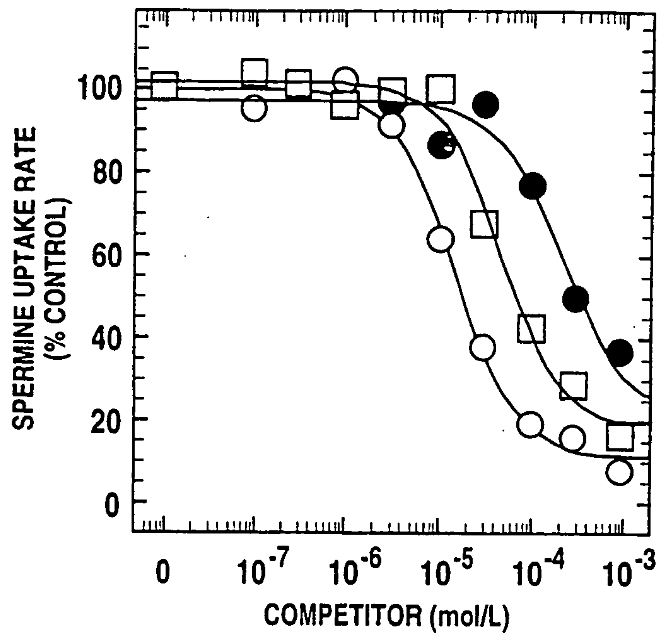


Fig. 2

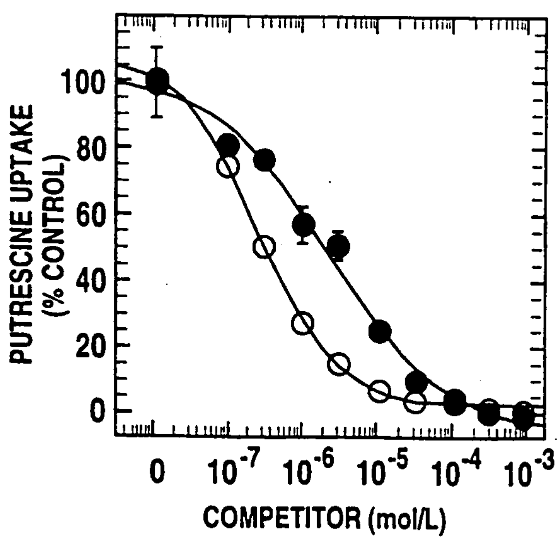


Fig. 3A

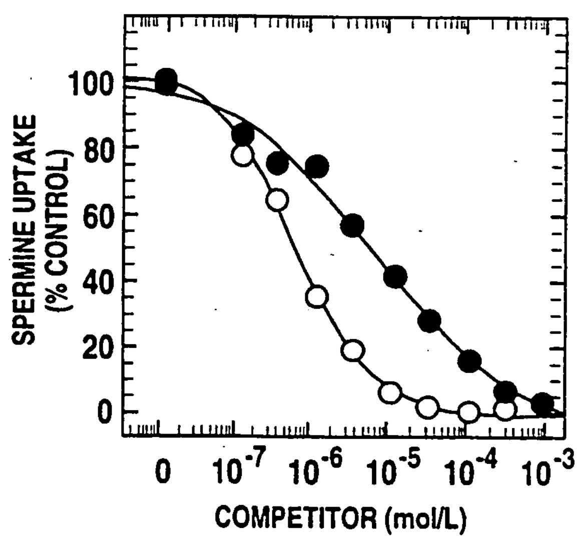
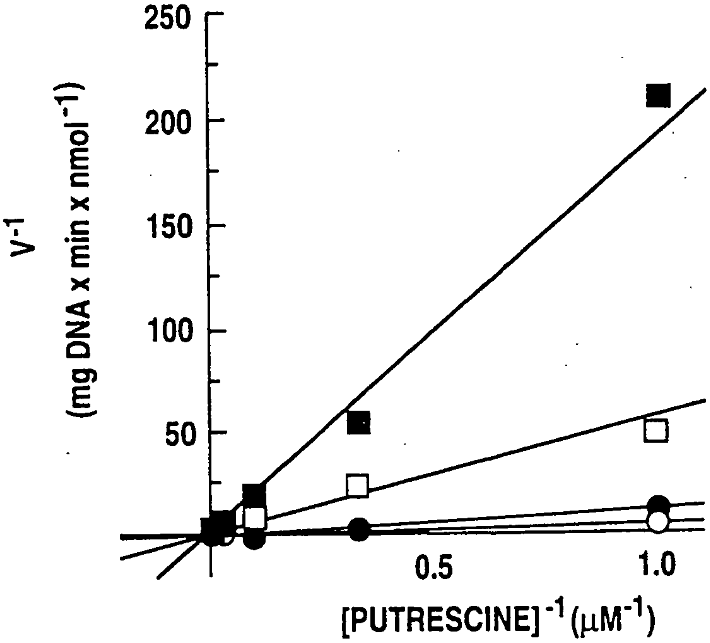
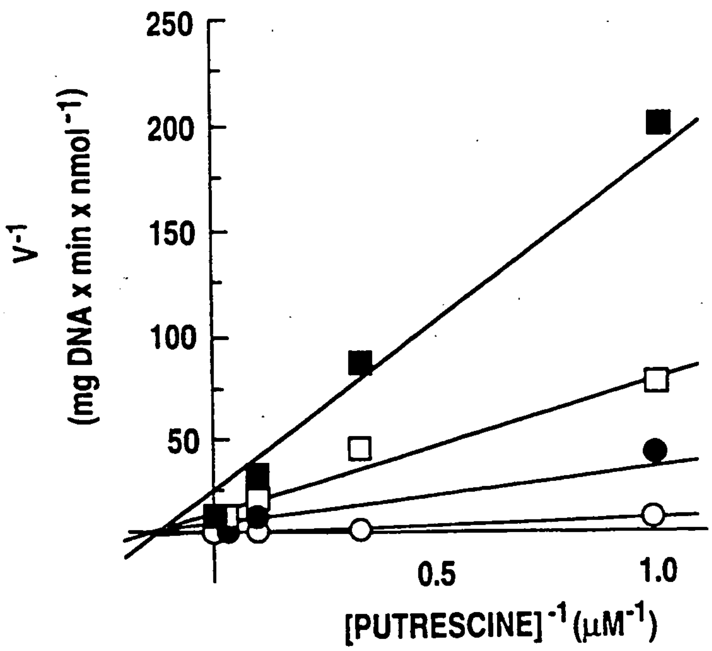


Fig. 3B



250
200
150
100
50
V-1
(mg DNA x min x nmol-1)
0.5
1.0
[PUTRESCINE]-1 (μM-1)

Fig. 4A



250
200
150
100
50
V-1
(mg DNA x min x nmol-1)
0.5
1.0
[PUTRESCINE]-1 (μM-1)

Fig. 4B

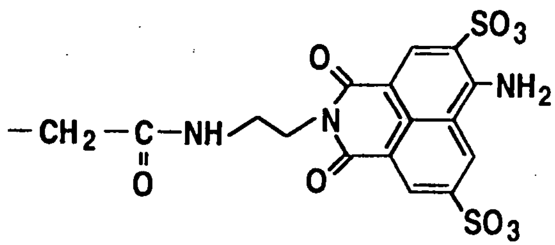
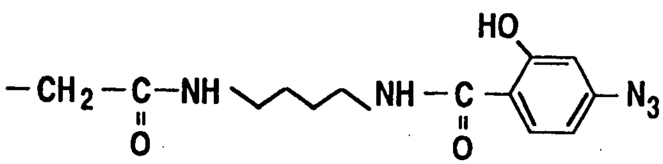
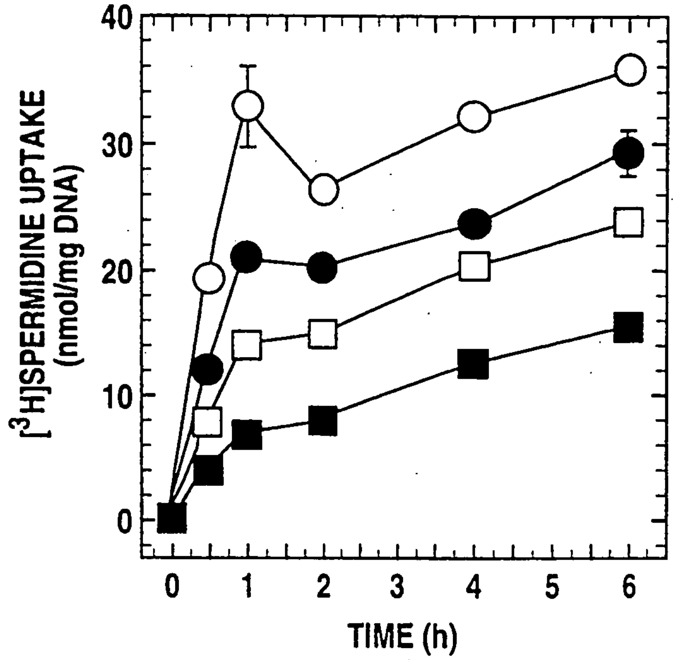
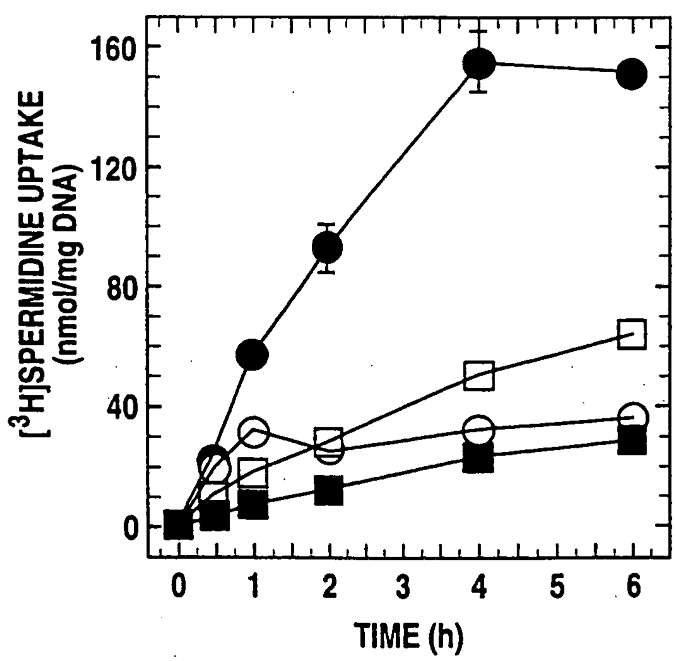
R	NAME	K_i (μM)
H	MESC	33.6±7.2
$-CH_2-C(=O)-NH_2$	MESC-iodoacetamide	48.9±9.1
	MESC-LY	44.1±8.8
	MESC-ASIB[a]	18.3±8.2

Fig. 5



[^{3}H]SPERMIDINE UPTAKE (nmol/mg DNA)
40
30
20
10
0
0
1
2
3
4
5
6
TIME (h)

Fig. 6A



[^{3}H]SPERMIDINE UPTAKE (nmol/mg DNA)
160
120
80
40
0
0
1
2
3
4
5
6
TIME (h)

Fig. 6B

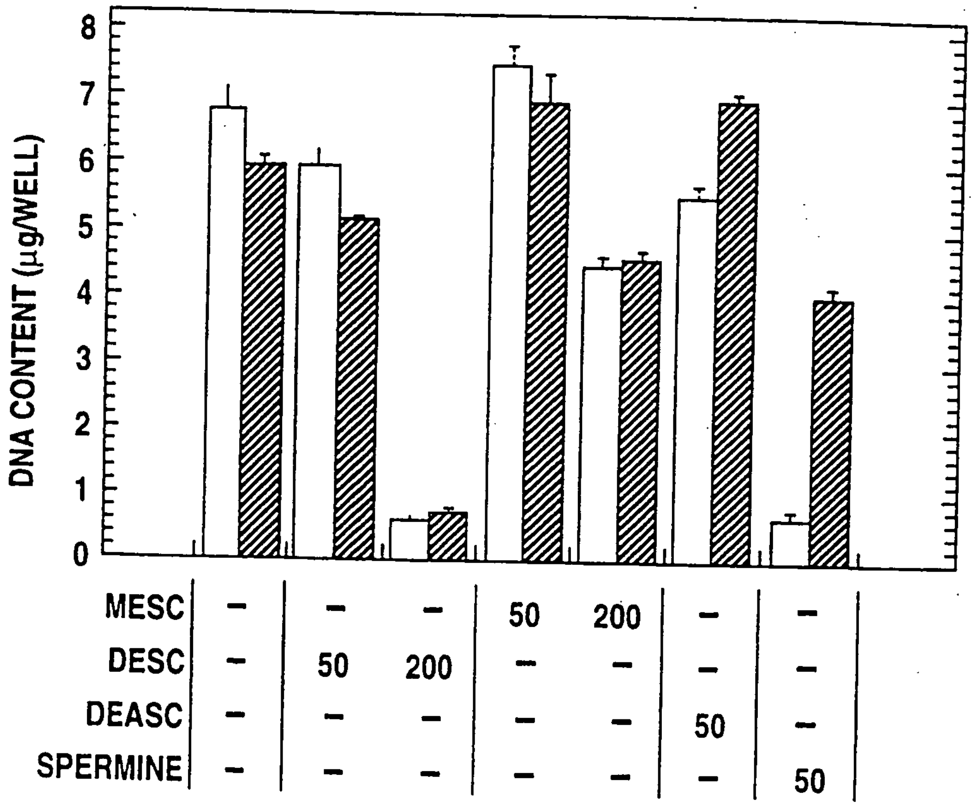

DNA CONTENT (μg/WELL)
8
7
6
5
4
3
2
1
0
MESC – – – 50 200 – –
DESC – 50 200 – – – –
DEASC – – – – – 50 –
SPERMINE – – – – – – 50

Fig. 7

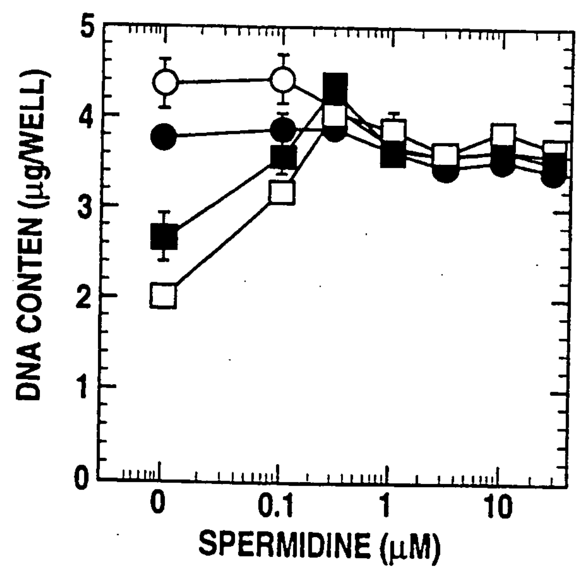

DNA CONTEN (μg/WELL)
5
4
3
2
1
0
0
0.1
1
10
SPERMIDINE (μM)

Fig. 8

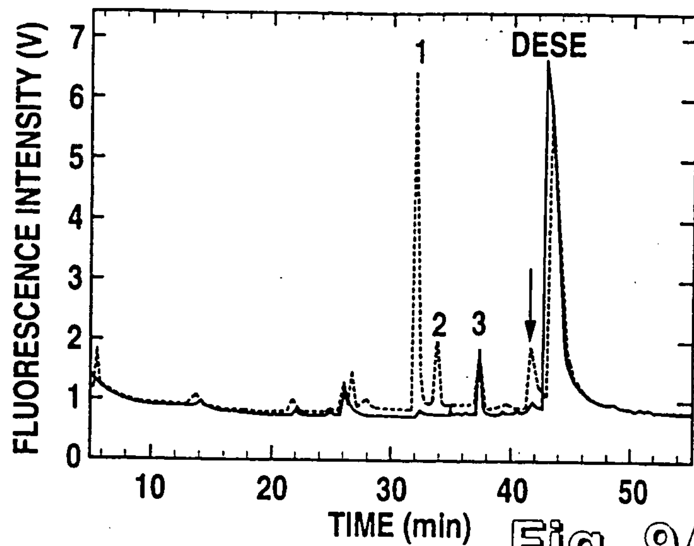


Fig. 9A

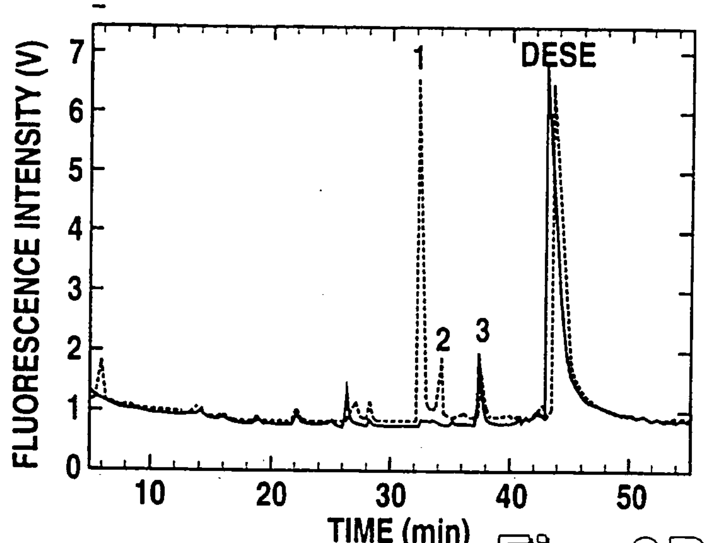


Fig. 9B

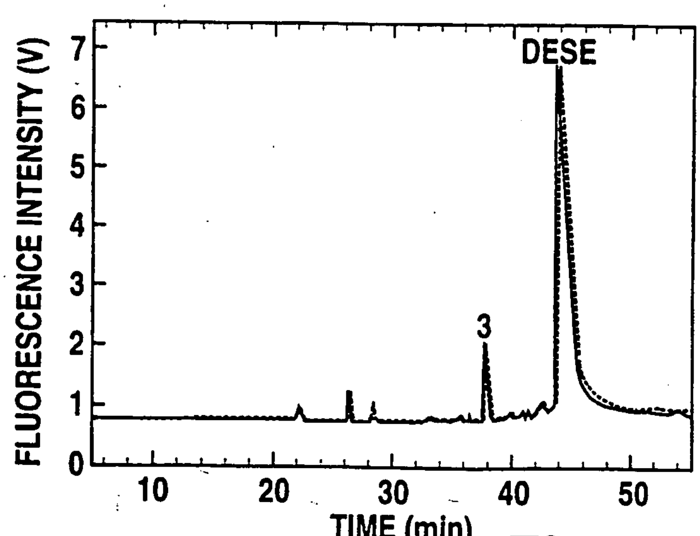


Fig. 9C

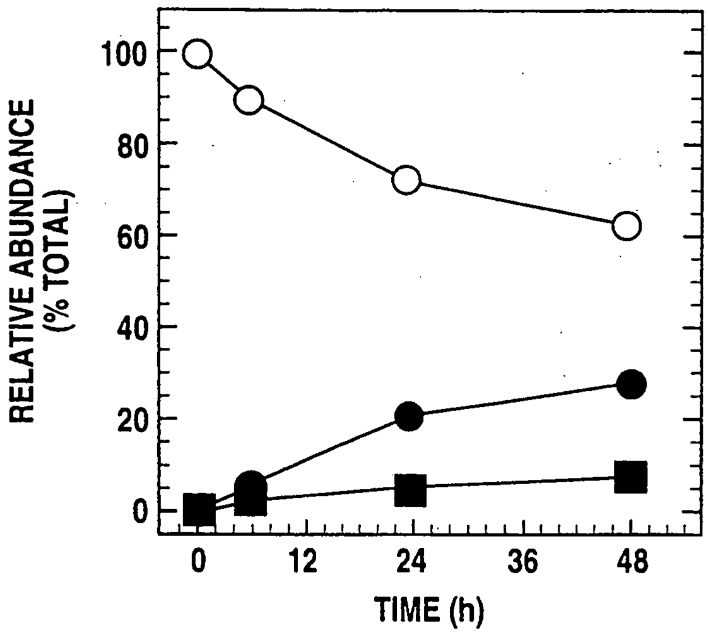


Fig. 10

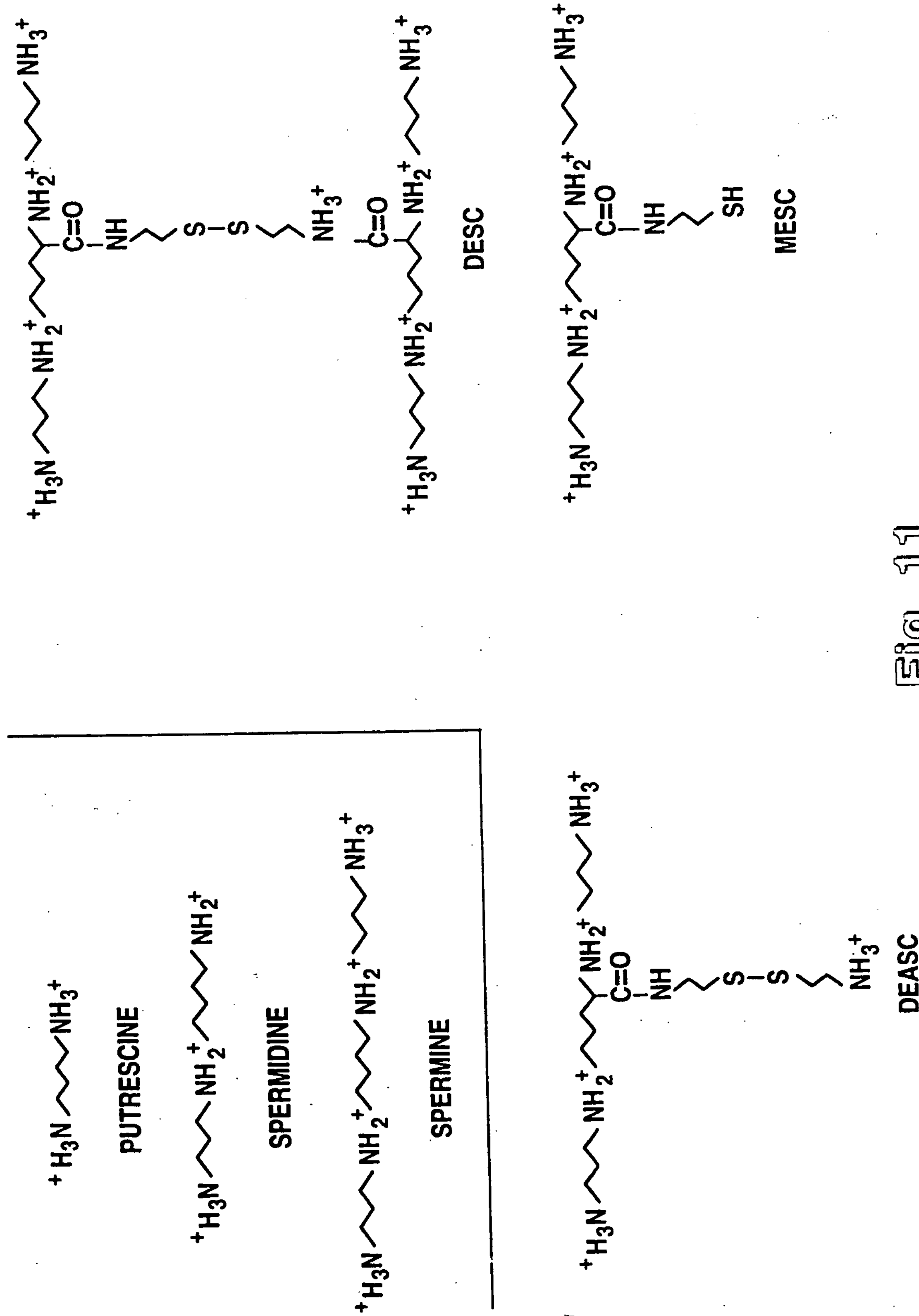


Fig. 11

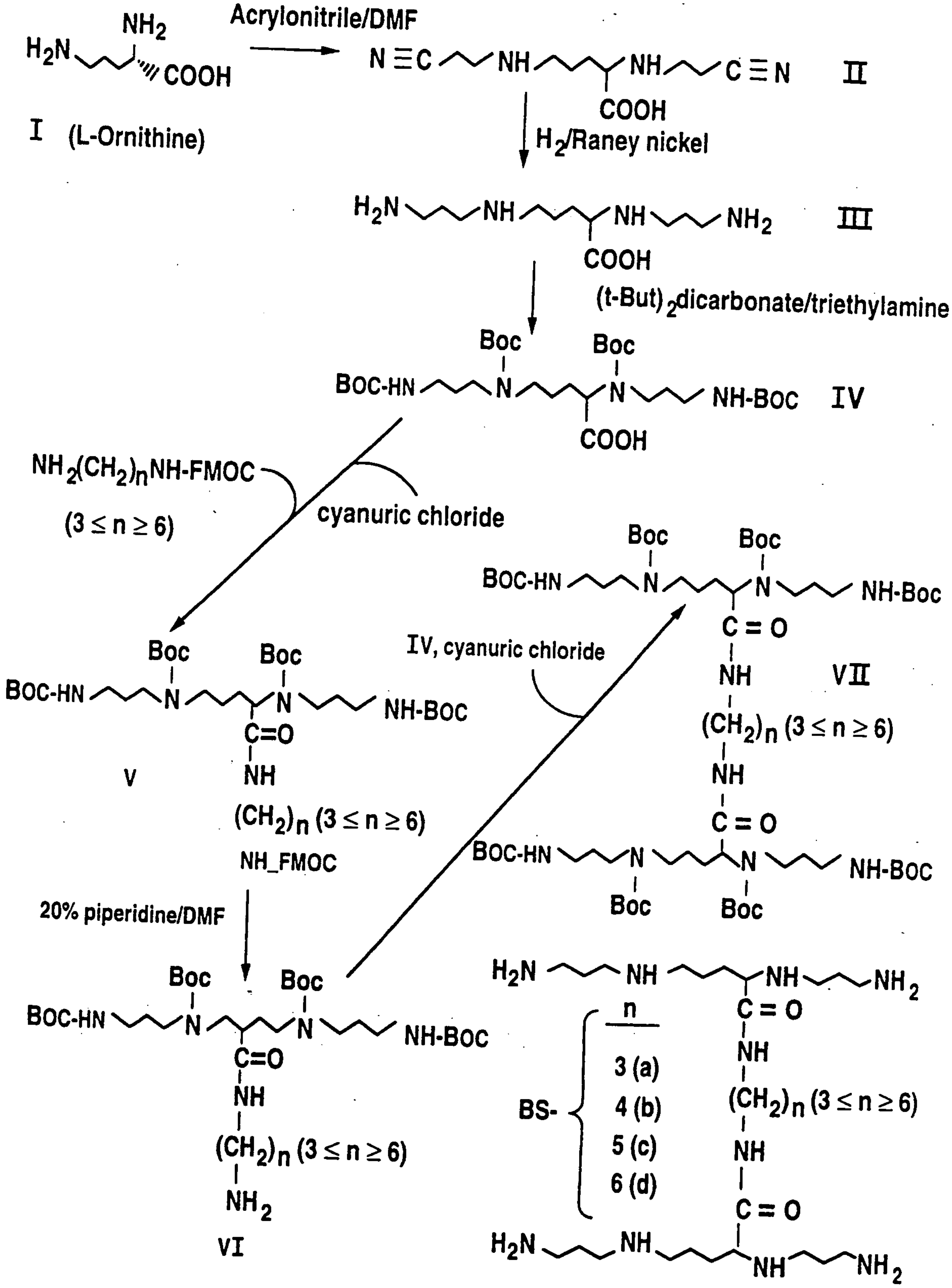

Acrylonitrile/DMF
I (L-Ornithine)
II
H2/Raney nickel
III
(t-But)2dicarbonate/triethylamine
IV
NH2(CH2)nNH-FMOC
(3 ≤ n ≥ 6)
cyanuric chloride
IV, cyanuric chloride
V
VII
20% piperidine/DMF
VI
BS-
n
3 (a)
4 (b)
5 (c)
6 (d)

Fig. 12

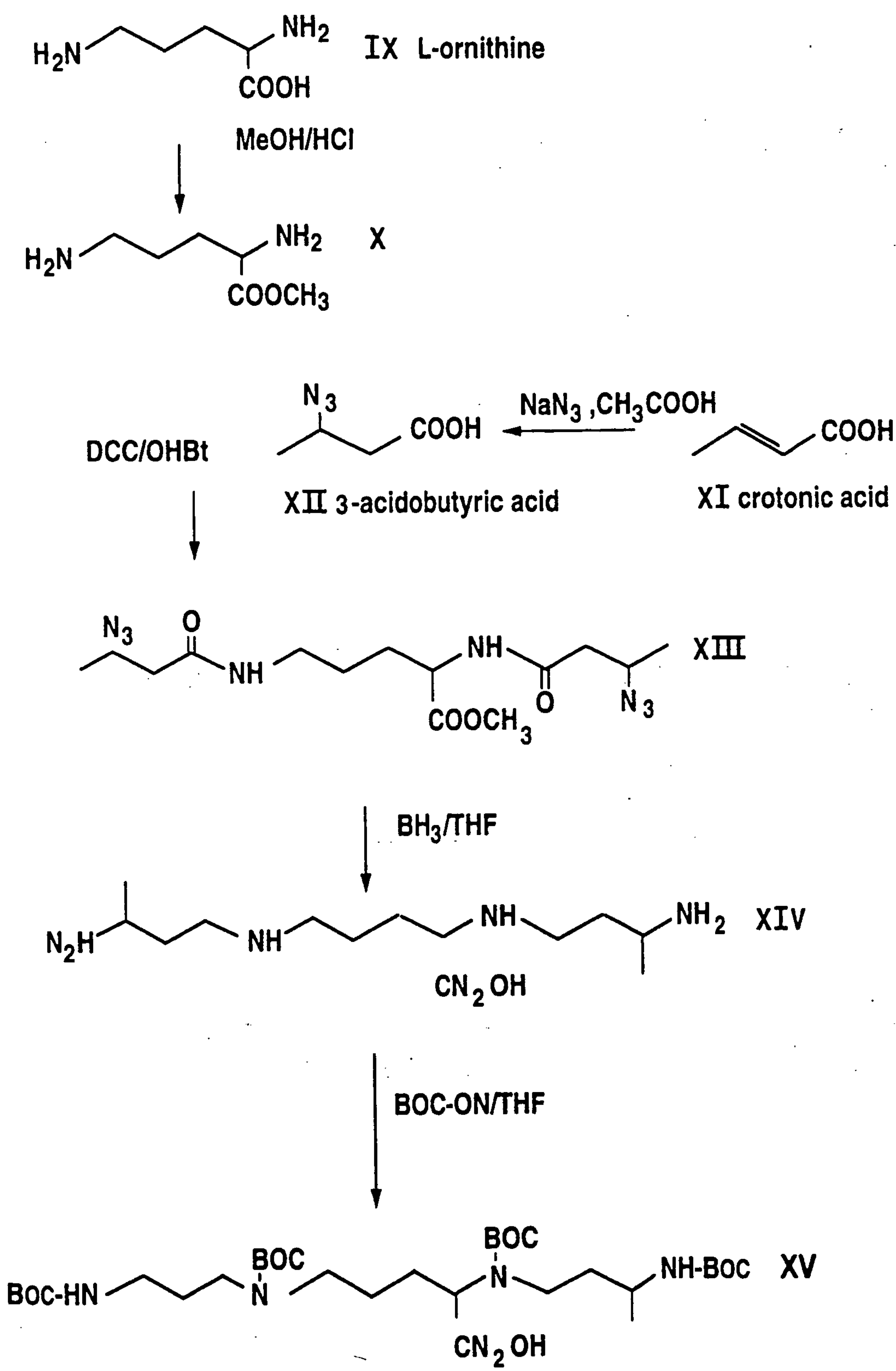

IX L-ornithine
MeOH/HCl
X
NaN3 ,CH3COOH
DCC/OHBt
XII 3-acidobutyric acid
XI crotonic acid
XIII
BH3/THF
XIV
BOC-ON/THF
XV

Fig. 13

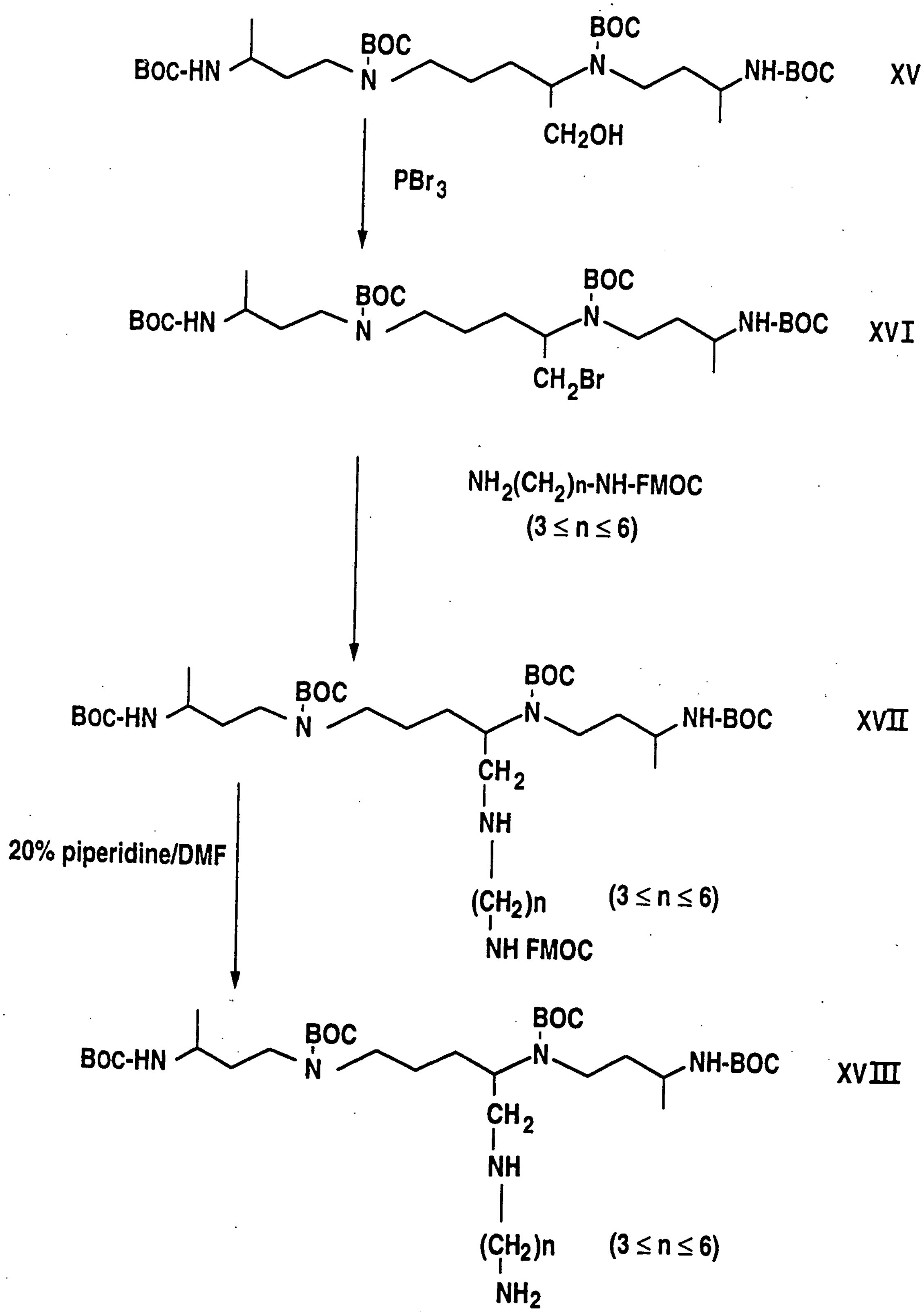
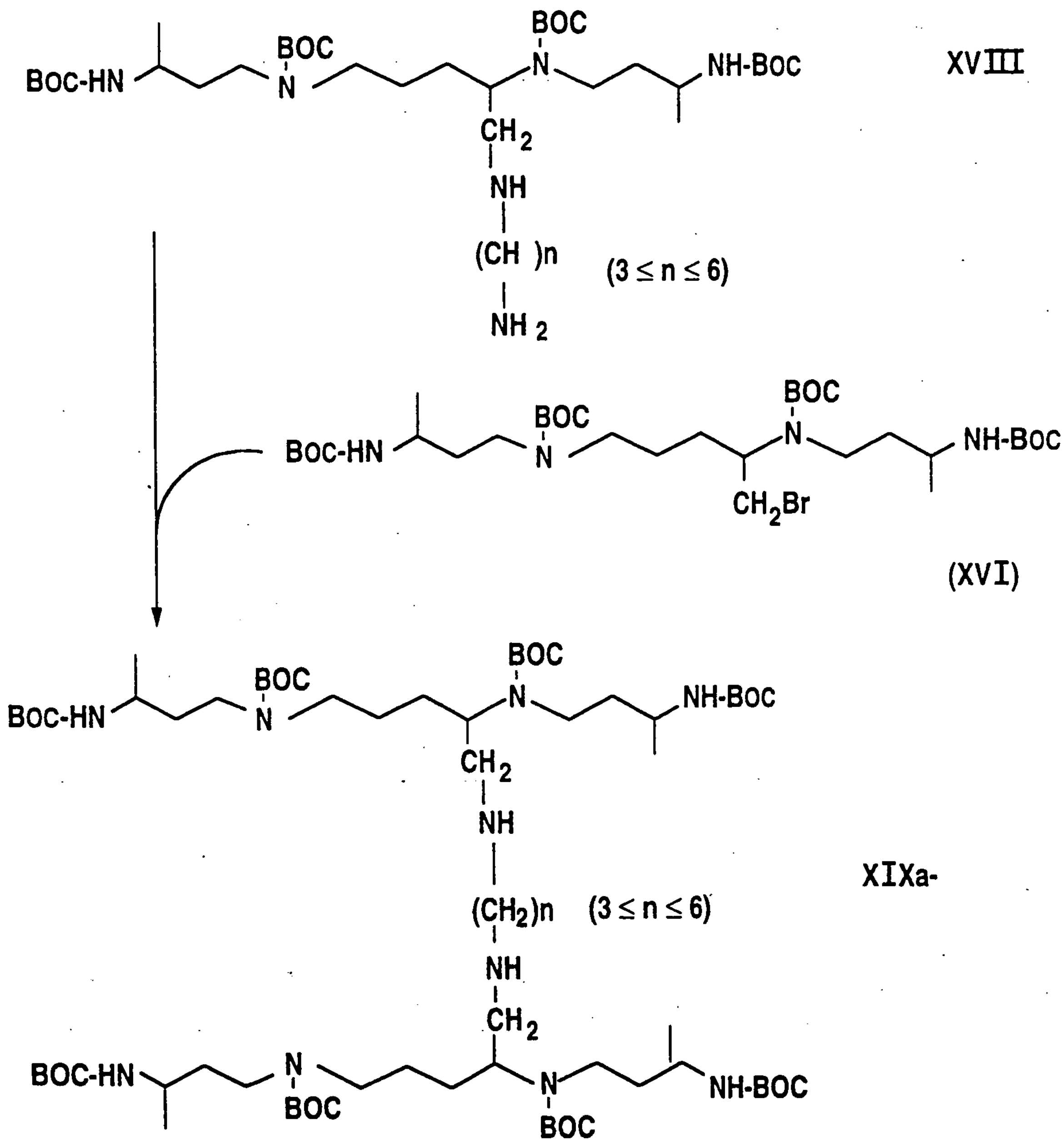

BOC-HN
BOC
N
BOC
N
NH-BOC
CH2OH
XV
PBr3
CH2Br
XVI
NH2(CH2)n-NH-FMOC
(3 ≤ n ≤ 6)
XVII
CH2
NH
(CH2)n
NH FMOC
20% piperidine/DMF
XVIII
NH2

Fig. 14

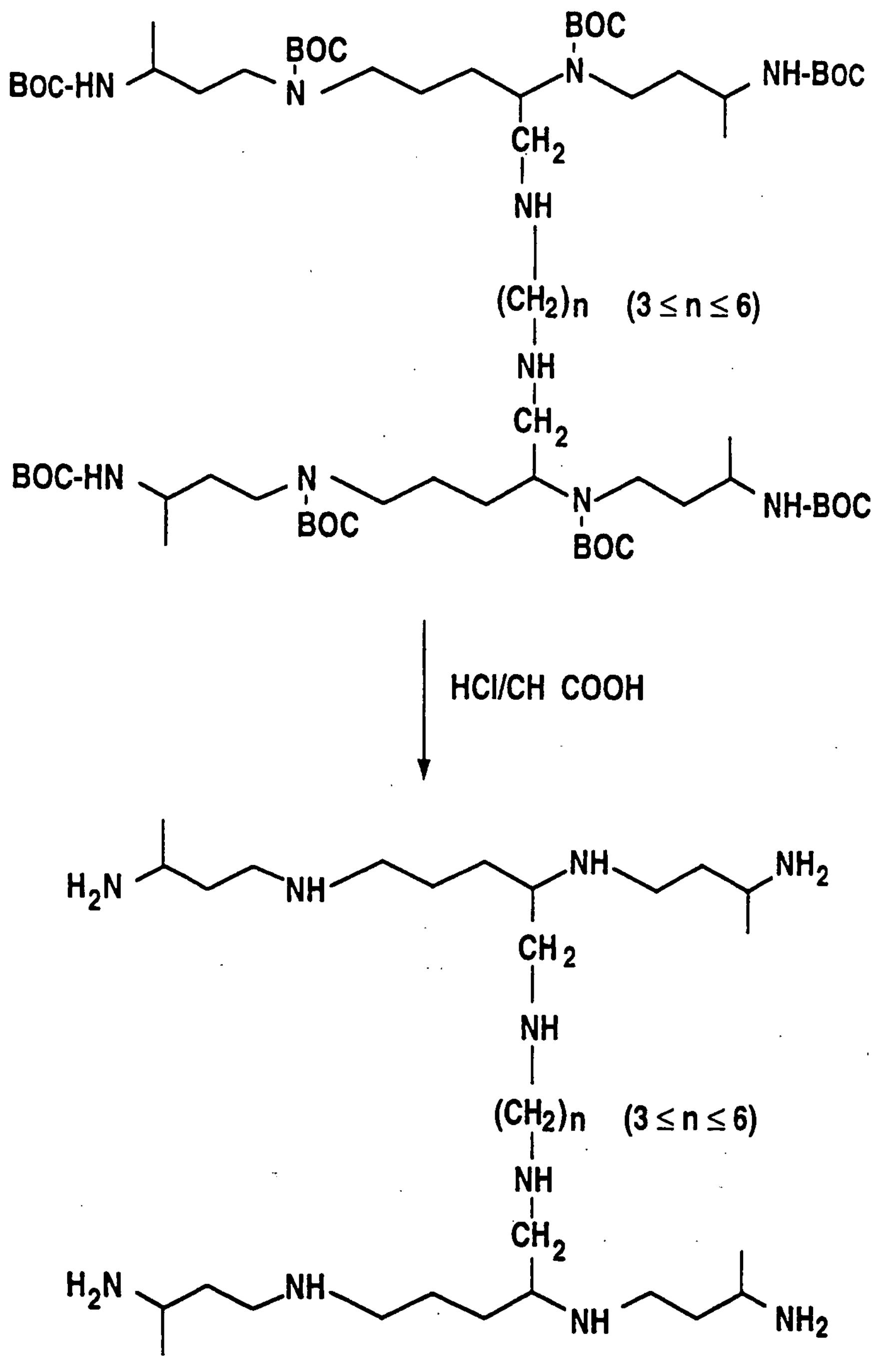


XVIII

(XVI)

XIXa-

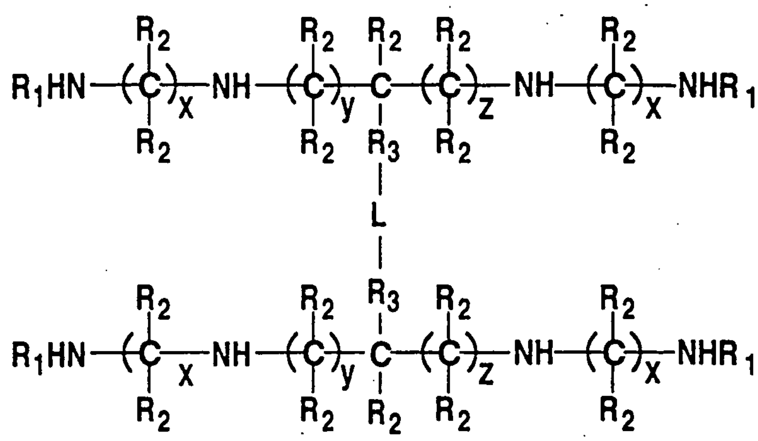
Fig. 15



XIXa-d

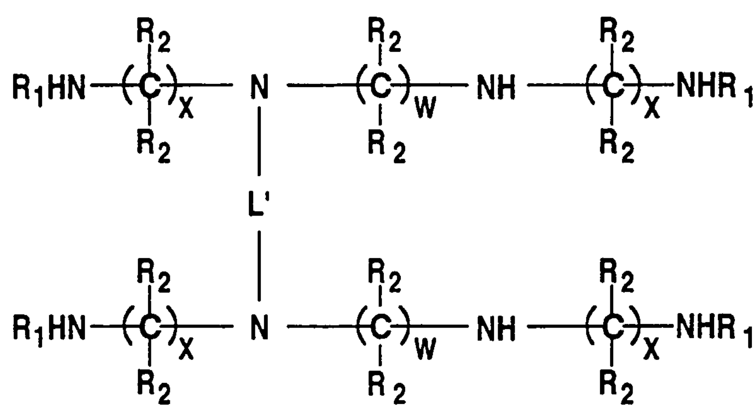
XXa-d

Fig. 16



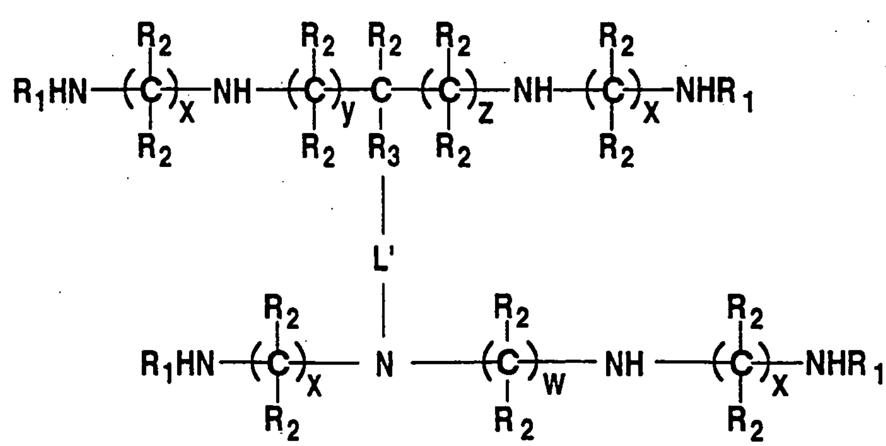
L = A CHEMICAL STRUCTURE (THE LINKER)

Fig. 17A



L' = A CHEMICAL STRUCTURE (THE LINKER)

Fig. 17B



L' = A CHEMICAL STRUCTURE (THE LINKER)

Fig. 17C

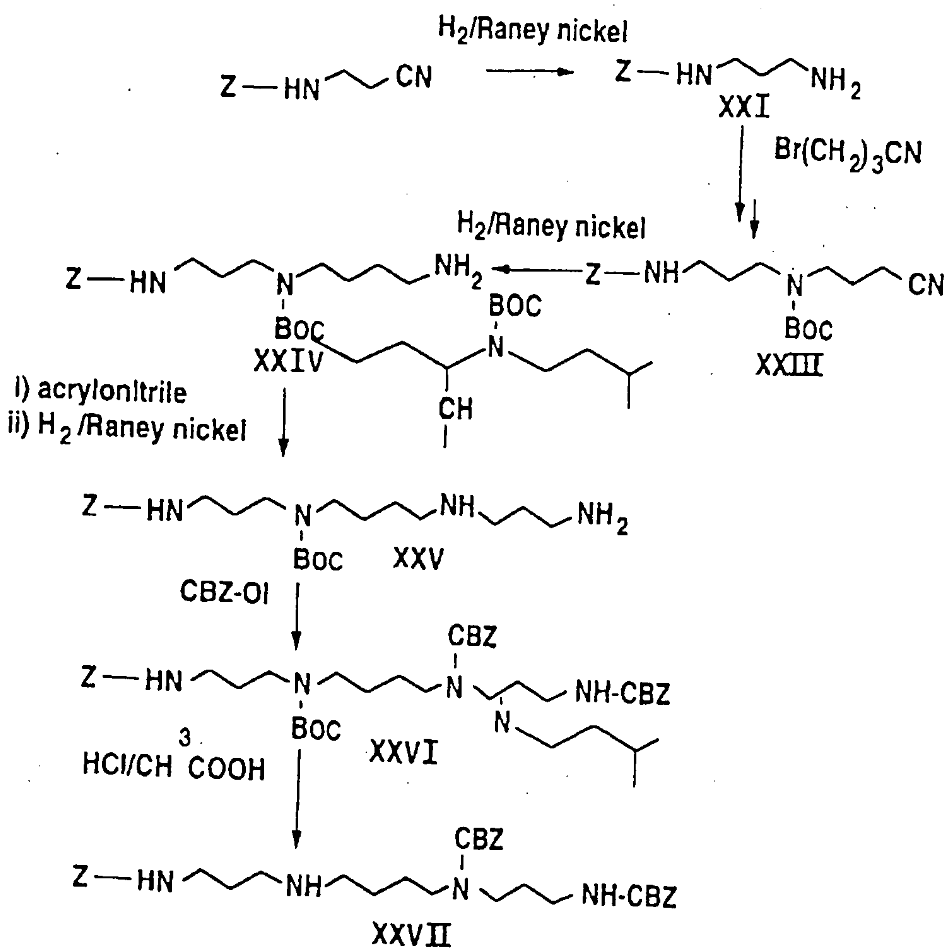

H2/Raney nickel
Z—HN CN
Z—HN NH2
XXI
Br(CH2)3CN
H2/Raney nickel
Z—HN N NH2
Boc
XXIV
Z—NH N CN
Boc
XXIII
BOC
N
CH
i) acrylonitrile
ii) H2/Raney nickel
Z—HN N NH NH2
Boc
XXV
CBZ-OI
CBZ
Z—HN N N NH-CBZ
Boc
N
XXVI
HCl/CH3COOH
CBZ
Z—HN NH N NH-CBZ
XXVII

Fig. 18

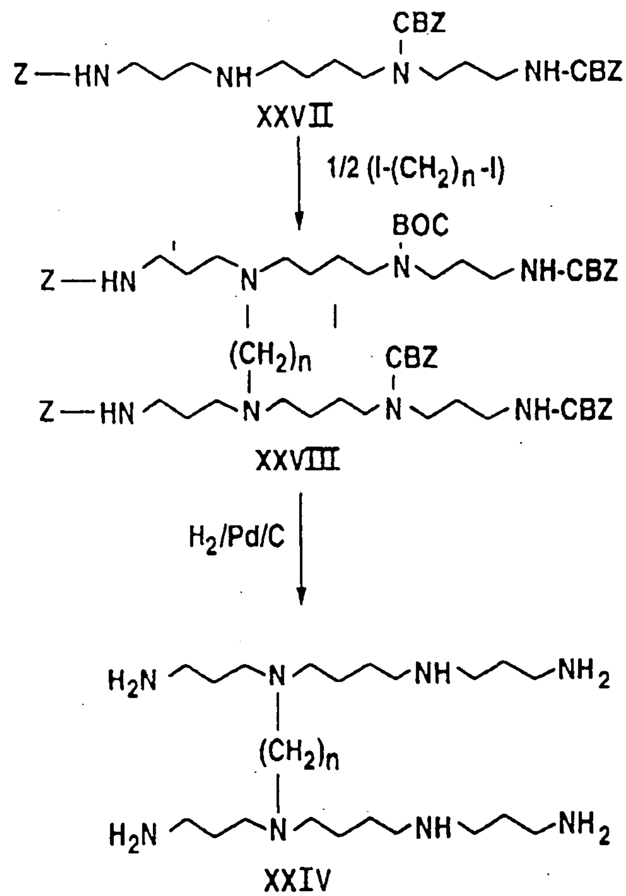
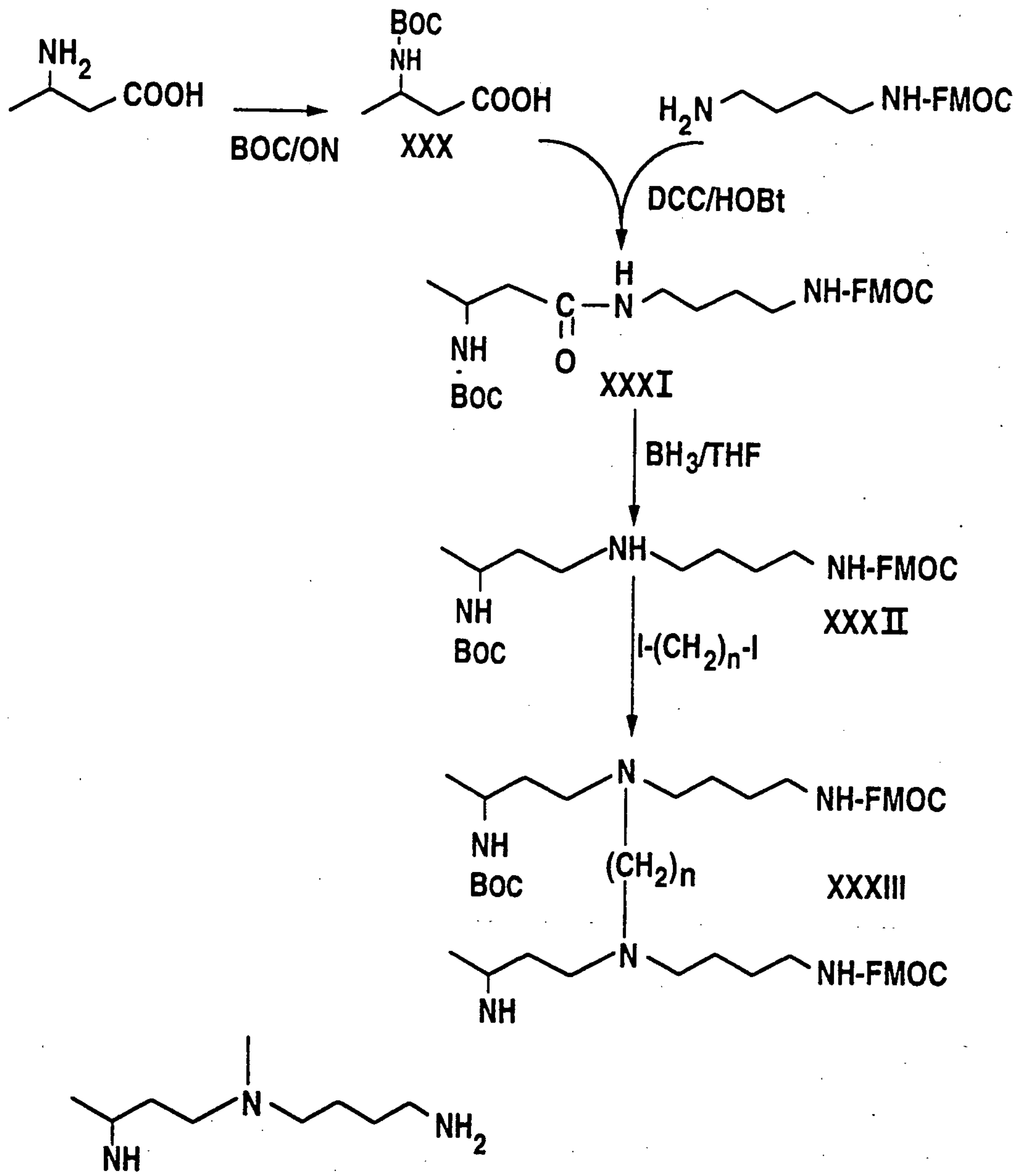
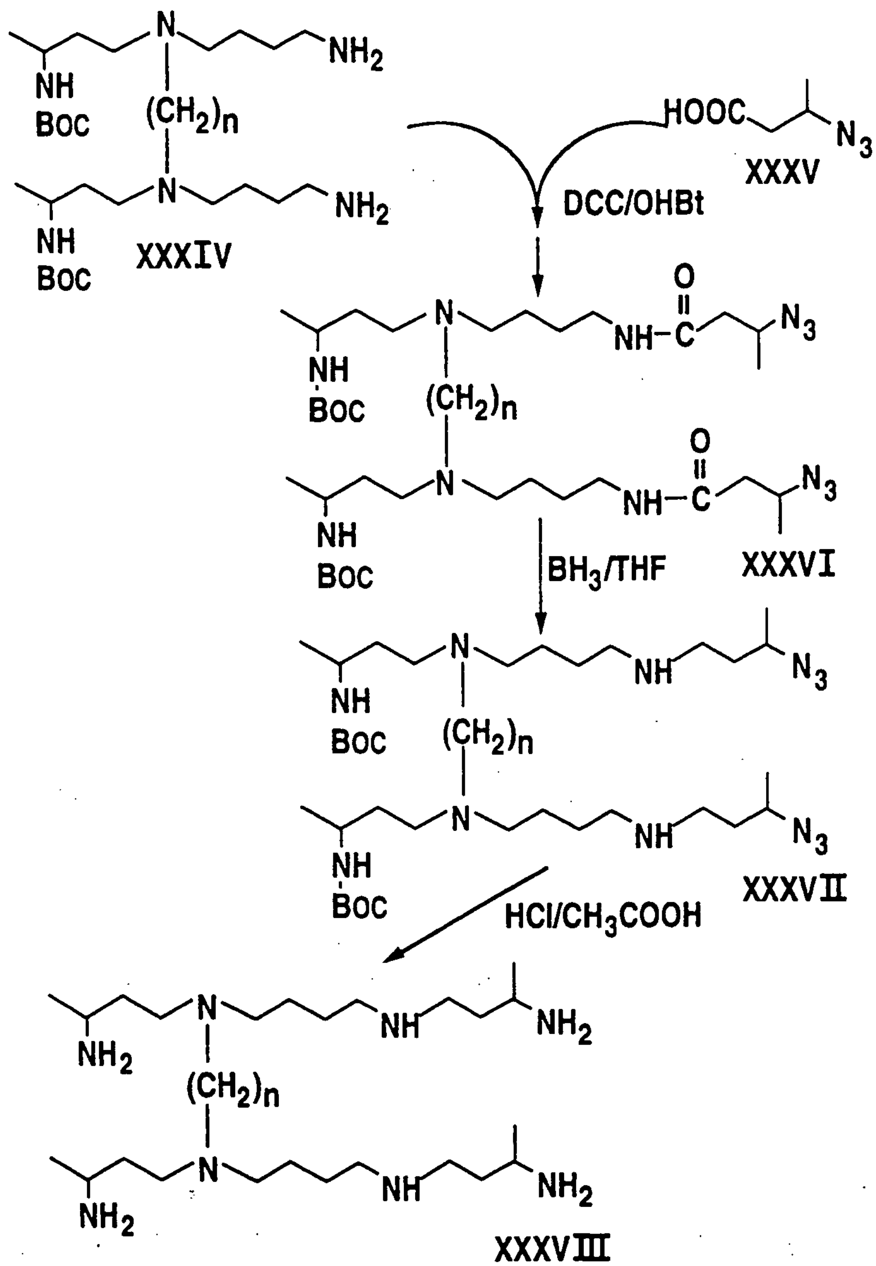

CBZ
Z—HN NH N NH-CBZ
XXVII
1/2 (I-(CH2)n-I)
BOC
Z—HN N N NH-CBZ
(CH2)n
CBZ
Z—HN N N NH-CBZ
XXVIII
H2/Pd/C
H2N N NH NH2
(CH2)n
H2N N NH NH2
XXIV

Fig. 19



NH2
COOH
BOC/ON
BOC
NH
COOH
XXX
H2N
NH-FMOC
DCC/HOBt
H
C
N
NH-FMOC
NH
O
BOC
XXXI
BH3/THF
NH
NH-FMOC
NH
BOC
XXXII
I-(CH2)n-I
N
NH-FMOC
NH
BOC
(CH2)n
XXXIII
N
NH-FMOC
NH
N
NH2
NH
BOC
XXXIV

Fig. 20



NH
Boc
$(CH_2)n$
NH_2
XXXIV
HOOC
N_3
XXXV
DCC/OHBt
NH–C
O
XXXVI
BH_3/THF
NH
XXXVII
HCl/CH_3COOH
XXXVIII

Fig. 21

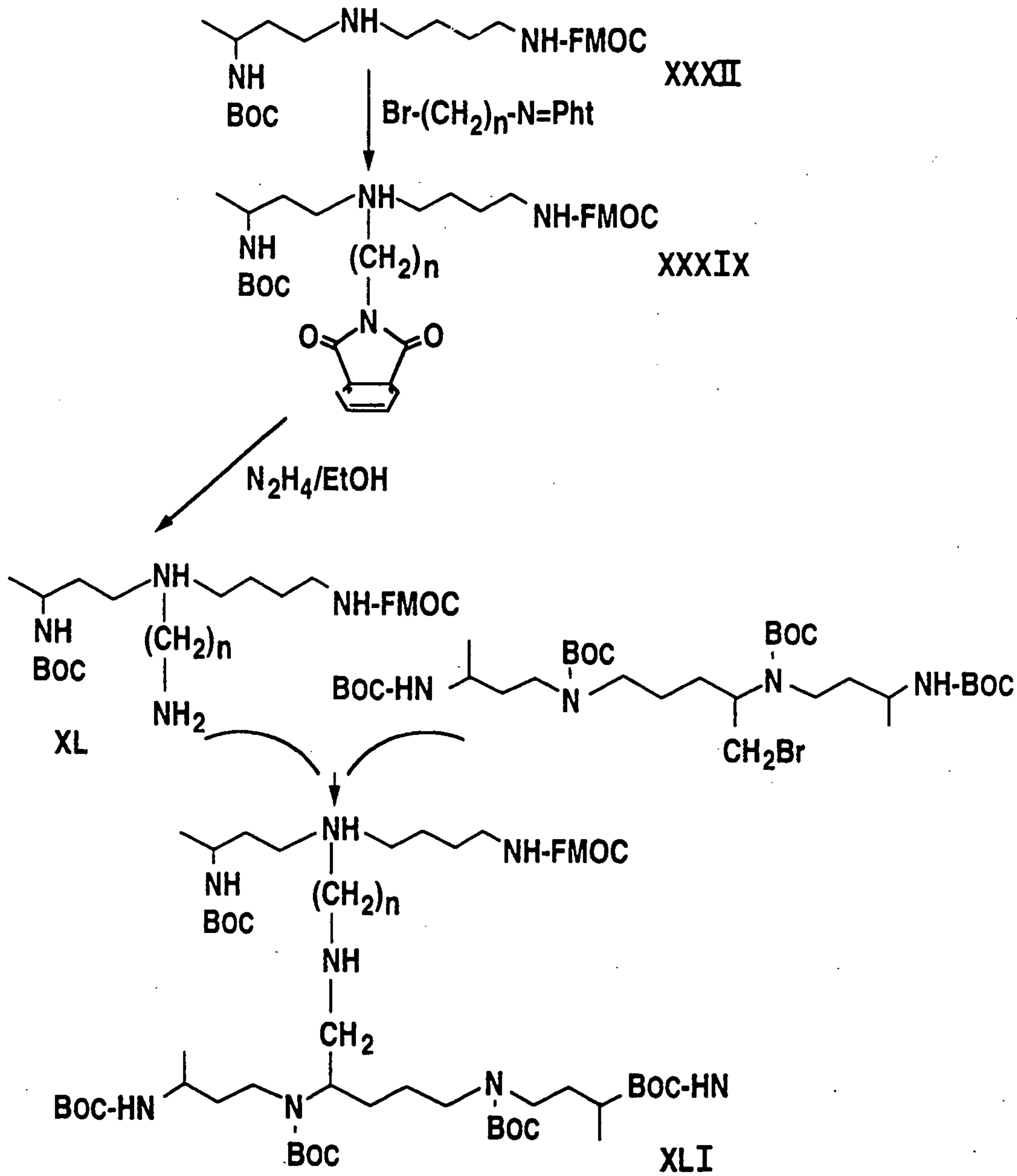

NH-FMOC
NH
BOC
XXXII
$Br-(CH_2)_n-N=Pht$
NH
NH-FMOC
NH
BOC
$(CH_2)_n$
XXXIX
O
N
O
$N_2H_4/EtOH$
NH
NH-FMOC
NH
BOC
$(CH_2)_n$
NH_2
XL
BOC
BOC
BOC-HN
N
N
NH-BOC
CH_2Br
NH
NH-FMOC
NH
BOC
$(CH_2)_n$
NH
CH_2
BOC-HN
N
N
BOC-HN
BOC
BOC
XLI

Fig. 22

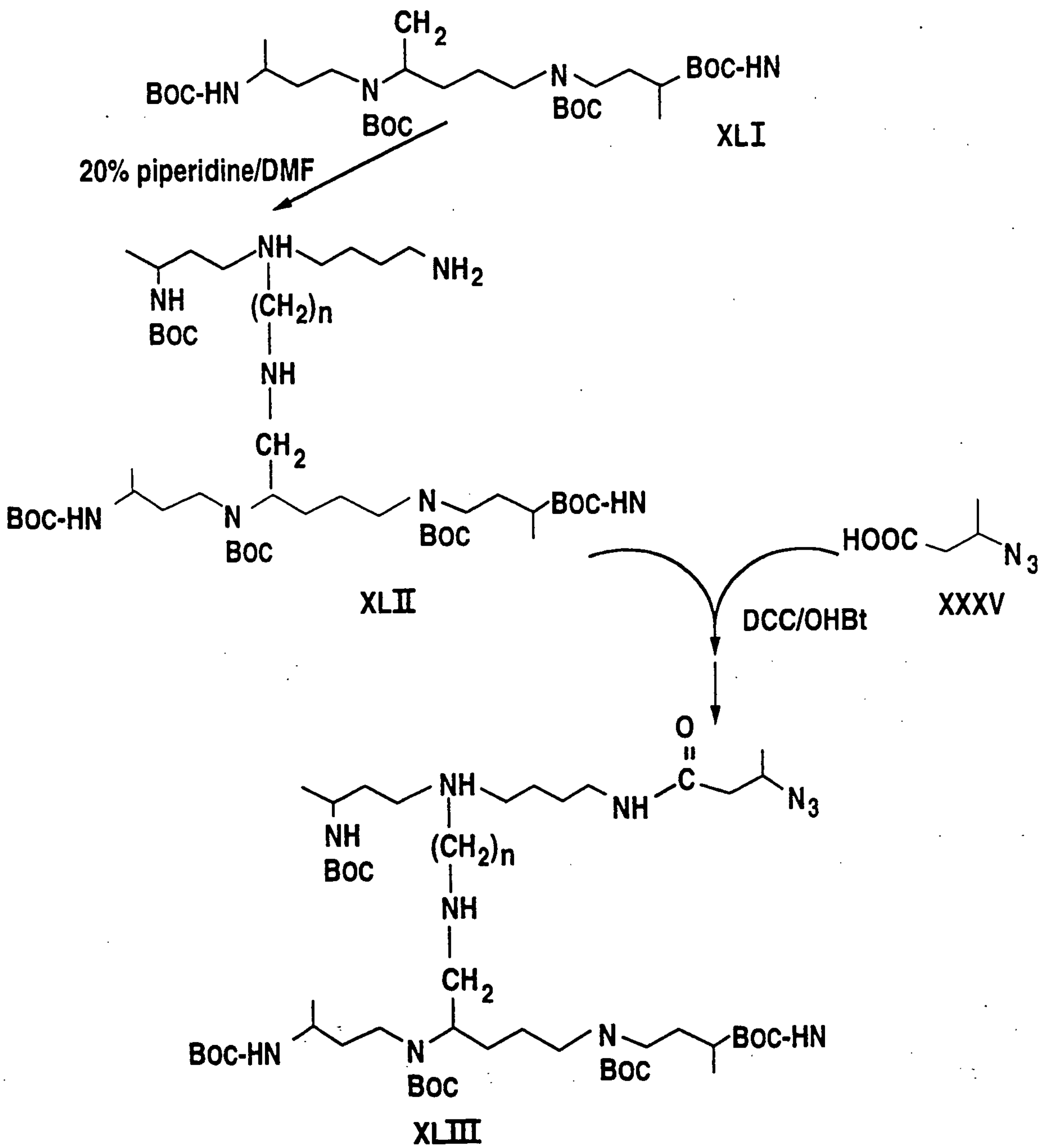
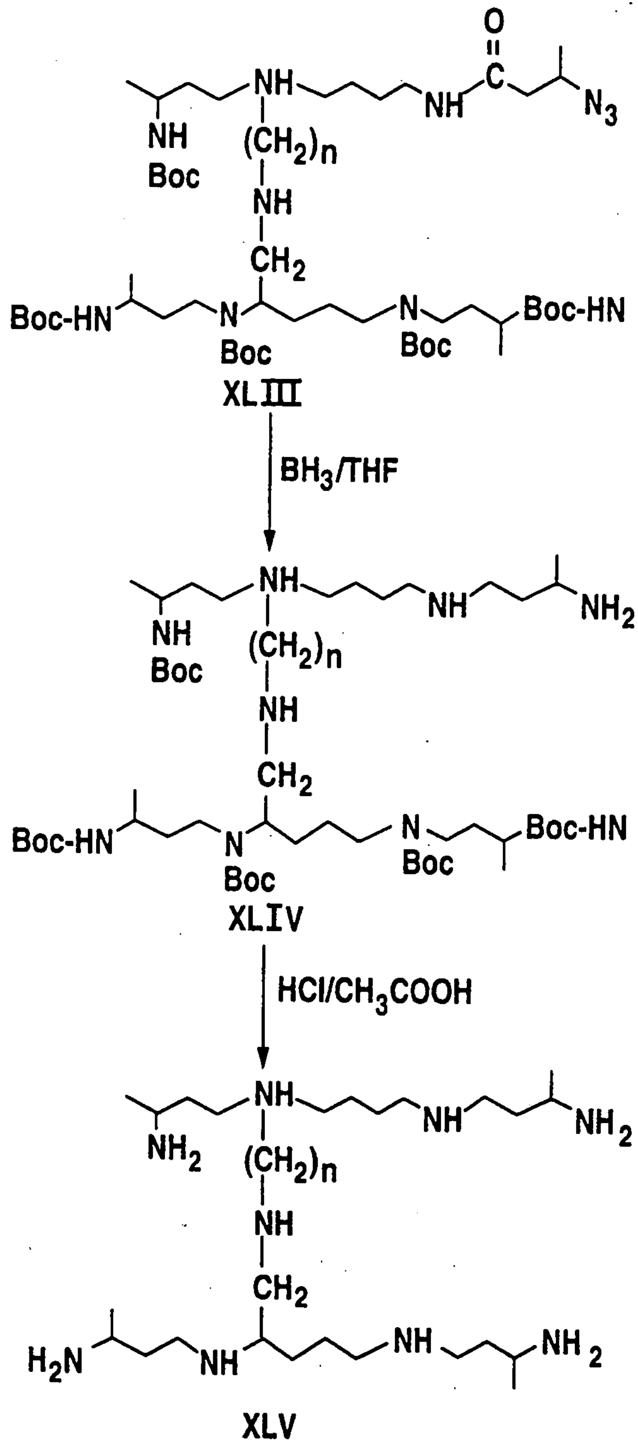

CH2
BOC-HN
N
BOC
N
BOC
BOC-HN
XLI
20% piperidine/DMF
NH
NH2
NH
BOC
(CH2)n
NH
CH2
BOC-HN
N
BOC
N
BOC
BOC-HN
XLII
HOOC
N3
XXXV
DCC/OHBt
O
C
NH
NH
N3
NH
BOC
(CH2)n
NH
CH2
BOC-HN
N
BOC
N
BOC
BOC-HN
XLIII

Fig. 23



O
C
NH
NH
N
N_3
Boc
$(CH_2)n$
CH_2
Boc-HN
Boc
XLIII
BH_3/THF
NH_2
XLIV
HCl/CH_3COOH
H_2N
XLV

Fig. 24